

AD \_\_\_\_\_

Award Number: W81XWH-11-1-0797

TITLE: Matrix metalloproteinases as a therapeutic target to improve neurologic recovery after spinal cord injury

PRINCIPAL INVESTIGATOR: Linda Noble

CONTRACTING ORGANIZATION: University of California at San Francisco  
San Francisco, CA 94118-6215

REPORT DATE: October 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

**REPORT DOCUMENTATION PAGE***Form Approved  
OMB No. 0704-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

**1. REPORT DATE**

October 2013

**2. REPORT TYPE**

Annual

**3. DATES COVERED**

30September2012-29September2013

**4. TITLE AND SUBTITLE** Matrix metalloproteinases as a therapeutic target to improve neurologic recovery after spinal cord injury

**5a. CONTRACT NUMBER****5b. GRANT NUMBER**

W81XWH-11-1-0797

**5c. PROGRAM ELEMENT  
NUMBER****6. AUTHOR(S)**

Linda J. Noble, Alpa Mahuvakar, Thomas Fandel, Aida F. Martinez, and  
Jon Levine

**5d. PROJECT NUMBER****5e. TASK NUMBER****5f. WORK UNIT NUMBER**

E-Mail: [linda.noble@ucsf.edu](mailto:linda.noble@ucsf.edu) jlevine@cvm.tamu.edu

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

The Regents of the University of California, Suite 315, Francisco  
3331 California Street  
San Francisco, CA 94118-6215

**8. PERFORMING ORGANIZATION  
REPORT NUMBER****9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S  
ACRONYM(S)****11. SPONSOR/MONITOR'S  
NUMBER(S)****12. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

#### 14. ABSTRACT

**Purpose:** We are evaluating efficacy of GM6001, a matrix metalloproteinase (MMP) inhibitor in a murine model of spinal cord injury (UCSF) and in dogs (Texas A & M, TAMU) that sustain naturally occurring spinal cord injuries resulting from spontaneous intervertebral disk herniation (IVDH).

**Scope:** These studies focus on efficacy of GM6001 in the context of an optimal therapeutic window and dependency on injury severity, using clinically relevant neurologic and urologic outcome measures.

**Major findings:**

-Spinal cord injury (SCI) in mice resulted in marked injury severity-dependent changes in locomotor and bladder function.

- GM6001 has an extended therapeutic window. When given up to at least 8 hours post injury, GM6001 resulted in injury severity dependent efficacy in a murine model of SCI. GM6001 treatment resulted in both neurologic and urologic benefit after a moderate level of SCI and was associated with a decrease in lesion volume and greater spared white matter volume. In contrast, GM6001 did not rescue locomotor or bladder function in mice with severe SCIs.

- Pharmacokinetic study of GM6001 in 10 dogs supports the short-term safety of the drug. Plasma drug levels following a single dose are sustained at a significant level likely to result in MMP inhibition for approximately 72 hours, which support a single dose strategy in the clinical trial.

-Developed cystometry protocol on 10 uninjured dogs.

-Enrolled 17 dogs with acute IVDH-associated SCI into serial cystometry study. Dogs with SCI that are non-ambulatory lack normal voiding reflex, have larger bladder capacity, elevated post-cystometry baseline pressure, and larger residual volume compared to measures taken during recovery.

**Significance:** We have found that GM6001 is efficacious when the therapeutic window is extended up to at least 8 hours after SCI of moderate severity. However, a similar benefit is not seen after a more severe SCI. The extended therapeutic window offers greater opportunity for translation to the theater for those soldiers who have sustained moderate SCIs. Pharmacokinetics of GM6001 in the dog is a key step in determining timing and dosing. Thus, we are now in an optimal position to translate this effort to a more naturally occurring SCI in dogs. Validation of GM6001 in two species would be a powerful argument for advancing this drug to human clinical trials.

#### 15. SUBJECT TERMS

spinal cord injury, matrix metalloproteinase inhibitor, intervertebral disk herniation, mouse, dogs, urologic function, neurologic function

#### 16. SECURITY CLASSIFICATION OF:

a. REPORT  
U

b. ABSTRACT  
U

c. THIS PAGE  
U

#### 17. LIMITATION OF ABSTRACT

UU

#### 18. NUMBER OF PAGES

92

#### 19a. NAME OF RESPONSIBLE PERSON USAMRMC

#### 19b. TELEPHONE NUMBER (include area code)

## Table of Contents

	<u>Page</u>
Introduction.....	1
Body.....	1-10
Key Research Accomplishments.....	11
Reportable Outcomes.....	12
Conclusion.....	13
References.....	14
Appendices.....	15- 61
Supporting Data.....	62-92

## INTRODUCTION

The primary objectives of this research are to evaluate the efficacy of a general inhibitor of matrix metalloproteinases, GM6001, in both a murine model of spinal cord injury (SCI) and in dogs who have sustained a naturally occurring SCI resulting from the sudden rupture of an intervertebral disk. The study builds upon our earlier work in the murine model of SCI, which showed that GM6001 significantly improved neurologic outcome when given 3 hours post injury after a moderate SCI (1). Thus, the goal here was to determine if GM6001 is likewise efficacious if the window of therapeutic intervention is extended and if the injury is more severe. An additional objective was to determine if GM6001 improves bladder function. We tested the efficacy of GM6001 dissolved in DMSO when administered subcutaneously at 8 hours after either a moderate or severe injury in mice. GM6001 improved both neurologic and urologic outcomes in the moderately injured group but not in the more severely injured group. Findings from the mouse studies have served to inform the dog preclinical trial, where the focus has been on the initial categorization of dogs according to severity of injury and assessment of GM6001 efficacy as determined by both neurologic and urologic assessments.

Please note that each task, described below, is indicated in bold. The requested and approved changes are indicated in bold italics.

## BODY

### UCSF Site:

#### ***Specific Aim 1***

#### **Task 1. Refine the therapeutic window for GM6001 in mice**

##### **1a. Obtain animal use protocol approval to study 165 mice (months 1-4)**

We received approval from the UCSF IACUC and ACURO to conduct these studies.

##### **1b. Compare neurologic recovery in 30 mice when GM6001 is initiated at 8 hours post injury. (months 5-6)**

For reasons described below (Texas A & M, Specific Aim 2, Task 1b) GM6001 was not available until month 8<sup>th</sup> of this project. In the interim, we refined our murine SCI model so that we could reproducibly generate both moderate and severe SCIs (as required for Specific Aim 1, Task 2a) and defined a series of abnormal urologic parameters that are present after SCI including uninhibited bladder contractions and changes in peak bladder pressure, bladder volume, and bladder weight. These experiments have provided a foundation for Specific Aim 1, Task 3A. Finally, beginning in month 10, we began Task 1b. Below summarizes our findings.

To *confirm a reproducible, graded model of SCI*, male, C57Bl/6 mice were subjected to a 2 gm weight dropped 5 cm (mild injury), a 2 gm weight dropped 7.5 cm (moderate injury), or a 3 gm weight dropped 5 cm (severe injury) onto the cord exposed at the T 9 vertebral level (Figure 1). Severity was defined based upon the BMS scale where a score of 0 indicates hindlimb paralysis and a score of 9 reflects normal hindlimb locomotor function. The more mildly injured animals showed scores of about 7.5 (frequent to consistent stepping and mostly coordinated in their locomotion). The moderately injured group scored about 3.5 (occasional plantar stepping). The severely injured animals scored about a 2 (hindlimb movement limited to extensive ankle movement). Representative urodynamic tracings (Figure 2), resulting from awake cystometry in mice subjected to mild, moderate, or severe SCIs, revealed distinct differences between injury severities with mild injuries showing qualitatively the most prominent bladder contractions relative to the moderately and severely injured group.

We *initially addressed long-term urologic status after mild and moderate SCIs*, focusing on 4 measures- namely uninhibited bladder contractions (UICs), residual urine, bladder weight, and bladder volume. As might be expected mild and moderate injuries resulted in more prominent uninhibited bladder

contractions than sham controls (Kruskal–Wallis test followed by Dunn’s Multiple Comparison Test). (Figure 3, Upper Panel). Residual urine (Figure 3, Bottom Panel) was similar between mild SCIs relative to shams but was elevated in the moderately injured group relative to shams (Kruskal-Wallis followed by Dunn’s Multiple Comparison Test). In contrast, peak voiding pressure showed no differences between mild and moderate SCIs and sham groups (Figure 4). Finally, we analyzed bladder volumes (Figure 5) and bladder weights (Figure 6) using the same statistical approaches. While bladder weight increased incrementally after mild and moderate injury, bladder volume remained unchanged, relative to shams, after a mild injury, whereas significantly increased after a moderate injury.

In summary, we have successfully generated reproducible graded levels of injury severity based upon the BMS scale. Urologic status shows injury severity-dependent changes with UICs being most pronounced after a more mild injury than a moderate injury. We believe that reduced uninhibited bladder contractions with greater severity of SCI may reflect prolonged over distension of the bladder wall, which may damage the muscle layer. We further found greater residual urine, bladder volume and bladder weight in the moderate injured group relative to the sham controls. These findings suggest aberrant remodeling of the bladder wall, which could contribute to increased weight and reduced voiding.

*We next evaluated the efficacy of GM6001 when give 8 hours after a moderate SCI, using a blinded, randomized experimental design with a priori exclusion criteria.* A total of 25 C57Bl/6 adult male mice were subjected to a moderate SCI at T9. Two groups were studied: drug-treated (N= 12) or vehicle (carboxymethylcellulose)-treated (n= 13). At 8 hours post injury, all mice were evaluated using the BMS scale. Exclusion criteria were as follows: any animal showing an average score of > 0.5 at 8 hours post-injury or morbidity as defined in UCSF IACUC and ACURO approved protocols. GM6001, (100mg/kg, i.p.) was given at 8 hours post-injury followed by repeated administration every 12 hours for 3 days. The vehicle control group received the same timing/route of administration. Of the 12 GM6001 treated mice, a total of 5 were excluded from the study due to early death or early morbidity. Of 13 mice that were treated with vehicle, 4 were removed from study due to morbidity, 2 met exclusion criteria at 8 hours post injury, and 2 others had injury device malfunction. Thus, neurologic recovery was evaluated in N= 5 for the vehicle and N= 7 for drug. Two-way repeated measures ANOVA of the BMS score revealed the following: P= 0.58 for interaction, P<0.0001 for time, and P= 0.16 for treatment (Figure 7).

We then evaluated improvement between the groups by comparing initial BMS scores at day 1 relative to final BMS scores at day 35 (Figure 8). Based upon a Student T-test, the drug treated group shower greater improvement than the vehicle group (P= 0.025). Finally, since weight supported stepping is considered to be a very favorable outcome, we evaluated the percentage of mice that showed frequent stepping (Figure 9). Statistical comparisons (2-way ANOVA) were done on percentages that were transformed into arcsin values. Approximately 60% of mice, treated with GM6001, showed frequent stepping whereas only 40% achieved that degree of recovery in the vehicle treated group. Based upon a 2-way ANOVA there was a significant effect of both treatment (P= 0.017) and time (P= 0.015). Taken together, despite the small group sizes, the behavioral data generally support improved recovery in mice treated with GM6001.

Finally, we have analyzed a cohort of bladders from these animals by awake cystometry. While we saw no differences in bladder volume, bladder weight or residual urine, the GM6001 treated group showed a significant reduction in uninhibited bladder contractions, one of the key features of dyssynergia (Figure 10).

After approval from the UCSF IACUC and ACURO, ***we began a 2<sup>nd</sup> set of studies to assess efficacy of GM6001 using dimethyl sulfoxide (DMSO) as the vehicle and a subcutaneous (s.c) route of delivery. This change in experimental design was prompted by the design of the TAMU dog study, which required DMSO and s.c. drug delivery.***

#### **Task 1b. Compare neurologic recovery in 30 mice when GM6001 dissolved in DMSO and injected subcutaneously is initiated at 8 hours post injury. (Months 12-14)**

We evaluated the efficacy of GM6001 dissolved in DMSO when given s.c at 8 hours after injury, using a randomized, blinded design with a priori exclusion criteria.

A total of 32 C57Bl/6 adult male mice were subjected to a moderate SCI at T9- drug (N= 16) and vehicle (n= 16). At 8 hours post injury, all mice were evaluated using the BMS scale. Exclusion criteria were as follows: any animal showing an average score of > 0.5 at 8 hours post-injury (n=6) or morbidity as defined in UCSF IACUC and ACURO approved protocols (n=3) or no locomotor recovery at 14 days post injury (n=1). GM6001 in DMSO was given s.q. at 100mg/kg at 8 hours post-injury followed by repeated administration every 12 hours for 3 days. The vehicle control group received the same timing/route of administration. Of the 16 GM6001 treated mice, 3 met exclusion criteria at 8 hours post-injury, 1 at 14 days post-injury and 1 had device malfunction. Of 16 mice that were treated with vehicle, 1 was removed from study due to morbidity, and 3 met exclusion criteria at 8 hours post injury. Thus, neurologic recovery was evaluated in N= 12 for vehicle and N=11 for drug group.

Neurologic recovery was measured using the Basso Mouse scale (BMS). Testing was performed at day 1, 3 and weekly thereafter for 5 weeks post-injury. Two-way repeated measures ANOVA of BMS scores revealed the following:  $P = 0.2453$  for interaction,  $P < 0.0001$  for time, and  $P = 0.0397$  for treatment (Figure 11). That is, all animals showed significant improvement in locomotor ability over time and there was a significant effect of drug on motor recovery. The average BMS score in the drug treated group at 35 days was 4.273 (able plantar step), whereas the vehicle treated group had an average BMS score at 35 days of 3.292 (unable to plantar step).

We next compared body weights over time in moderately injured mice treated with DMSO or GM6001. In response to injury, all mice lost weight over the first three days and gained weight thereafter. Importantly, drug treated mice gained more weight as compared to vehicle treated mice (Two-way repeated measures ANOVA of body weight revealed the following:  $P = 0.0676$  for interaction,  $P < 0.0001$  for time, and  $P = 0.0104$  for treatment) (Figure 12).

Taken together, these findings serve as the 3<sup>rd</sup> independent study to validate GM6001 as a therapeutic for SCI. We have shown that when used in combination with the vehicle methylcellulose and given i.p., GM6001 improves long-term neurological recovery when treatment is initiated at either 3 or 8 hours post injury. In task 1b, we confirm efficacy using a different route of administration (s.c.) and a different vehicle (DMSO).

### **1c. Compare neurologic recovery in 30 mice when GM6001 is initiated at 6 or 12 hours depending on the results 1b. (Months 7-8)**

*We received permission from the Grants Office's Representative to eliminate task 1c, so that we could repeat task 1b, testing a subcutaneous route of administration. Please see above for the repeat dosing at 8 h with DMSO as vehicle and subcutaneous route of injection for moderate level injury severity.*

### **Task 2. Determine if GM6001 will be efficacious after a more severe SCI in mice.**

#### **2a. Compare neurologic recovery in 30 mice after a severe SCI. (Months 16-18)**

We evaluated the efficacy of GM6001, dissolved in DMSO, when given subcutaneously at 8 hours after severe SCI, using a blinded, randomized design with a priori exclusion criteria. A total of 31 C57Bl/6 adult male mice were subjected to a severe contusion injury at T9- Drug (N= 16) and vehicle (N= 15). At 8 hours post injury, all mice were evaluated using the BMS scale. Exclusion criteria were as follows: any animal showing an average score of > 0.5 at 8 hours post-injury or morbidity as defined in UCSF IACUC and ACURO approved protocols (n=2). GM6001 (100 mg/kg), dissolved in 99% DMSO, was given s.c. at 8 hours post-injury followed by repeated administration every 12 hours for 3 days. The vehicle control group received the same timing/route of administration. Of the 16 GM6001 treated mice, 1 had device malfunction and 3 met the morbidity criteria. Of 15 mice that were treated with vehicle, 2 were removed from study due to morbidity. Thus, neurologic recovery was evaluated in N= 12 for vehicle and N=13 for drug group.

Neurologic recovery was measured using the Basso Mouse scale (BMS). Testing was performed at day 1, 3 and weekly thereafter for 5 weeks post-injury. Two-way repeated measures ANOVA of BMS score revealed the following:  $P = 0.9468$  for interaction,  $P < 0.0001$  for time, and  $P = 0.7530$  for treatment

(Figure 13). That is, all animals showed significant improvement in locomotor ability over time but there was no effect of drug on motor recovery. The average BMS score in both groups at 35 days was 2.6, which means extensive ankle movement.

Body weights were evaluated over time in severely injured mice treated with DMSO or GM6001. All animals gained weight with time, but there was no effect of drug treatment at this level of injury (Two-way repeated measures ANOVA:  $P = 0.2662$  for interaction,  $P < 0.0001$  for time, and  $P = 0.5362$  for treatment) (Figure 14).

### **Task 3. Determine if GM6001, when optimally delivered, will improve bladder function in mice.**

#### **3a. Compare urologic function in spinal cord injured mice treated with either vehicle or GM6001 after moderate and severe SCIs. (Months 14-20)**

At 5 weeks after a moderate or severe SCI in mice treated with GM6001 or DMSO, a PE10 catheter was implanted into the bladder dome and 2-3 days later awake cystometry was conducted. The following parameters were measured: time to first void, uninhibited bladder contractions/cycle, residual urine and voiding efficiency. Time to first void was defined as the period between when the saline infusion was initiated to the first release of fluid from the urethral meatus. To measure uninhibited bladder contractions (UIC), animals were first exposed to an equilibration period of 30 minutes during which the bladder was filled with saline. After this equilibration period, UICs were evaluated. UIC's were defined as rhythmic intravesical pressure rises ( $>5$  cm H<sub>2</sub>O from baseline pressure) without a release of fluid from the urethra using three representative voiding cycles. The numbers of non-voiding UICs per voiding cycle were determined. Residual urine was measured after the last void. The infusion was stopped and residual volume was determined by withdrawing the residual saline through the intravesical catheter. Voiding efficacy was calculated as (total infused volume – residual urine)/ total volume \* 100.

Cystometry results after moderate SCI. In the intact spinal cord, long descending fiber tracts from the midbrain coordinate the activity of the detrusor muscle and the urethral sphincter, i.e. the detrusor muscle is relaxed while the sphincter muscle is contracted to allow for filling of the bladder, while voiding is characterized by relaxation of the urethral sphincter and contraction of the detrusor muscle. After SCI, input from the brainstem is partially lost. As such, simultaneous contractions of detrusor and sphincter emerge. In cystometry, detrusor sphincter dyssynergia (DSD) is in part represented by detrusor contractions against a closed urethral sphincter without release of fluid (i.e. uninhibited bladder contractions).

Improved urodynamic outcomes were evident in GM6001 treated mice. That is, there was less urine retention (Unpaired two-tailed T- test,  $p = 0.0004$ ) and a decreased number of uninhibited bladder contractions per cycle (Mann Whitney test,  $p = 0.0067$ ). Bladder voiding was apparent at an earlier time point (Unpaired two-tailed T- test,  $p = 0.0004$ ), and mice had improved voiding efficacy (Mann Whitney test,  $p = 0.0075$ ) (Figure 15).

After SCI, the bladder undergoes extensive remodeling, characterized by collagen deposition and muscle hypertrophy. The end result of this remodeling is an increase in bladder weight. As expected, there was an increase in bladder weight in mice with moderate SCIs (Figure 16). Moreover, there was a strong trend associated with drug treatment group, whereby the increase in bladder weight appeared less than that seen in the vehicle treated group (Mann Whitney test,  $p = 0.0531$ ). Subsequent histological analysis was performed on these bladders ( $N = 6$  per treatment) within three regions (dome, middle and base). The thickness within each of these regions was significantly greater in the vehicle, relative to the drug-treated group (Mann Whitney test,  $p = 0.026$  for dome;  $p = 0.0022$  for middle;  $p = 0.0152$  for base) (Figure 17).

Cystometry results after severe SCI. We have found that severe SCI results in a characteristic leakiness of the bladder, which likely results from a highly over distended bladder that with time disrupts the detrusor muscle.



Drug treatment did not affect urine retention (Unpaired two-tailed T- test,  $p=0.6038$ ), time to first void (Unpaired two-tailed T- test,  $p=0.9573$ ), or voiding efficacy (Mann Whitney test,  $p=0.4401$ ) (Figure 18). However, it did result in a decreased number of UICs per cycle (Unpaired two-tailed T- test,  $p=0.0210$ ). We believe that reduced UICs may reflect prolonged over distension of the bladder wall, which may either damage the muscle layer or result in aberrant remodeling such that the muscle wall has reduced capability of contracting. Finally, we found that increased weight in the bladder was not attenuated by GM6001 (Unpaired two-tailed t-test,  $p=0.1390$ ) (Figure 19).

Collectively, these findings support the position that GM6001 shows injury severity dependent efficacy whereby moderate levels of SCI show both neurological and urological recovery.

#### **Task 4. Analysis of lesion epicenter and serotonergic fiber tracks caudal to a SCI in mice.**

##### **4a. Perfuse animals with fixative, remove the cords, and stain with Eriochrome cyanine or immunostain for serotonergic fiber tracks. (Months 5-24)**

All animals thus far studied have been perfused with fixative, cryoprotected, frozen and sectioned. We have stained and analyzed spinal cord sections from all the mice from moderate and severe injured groups with eriochrome cyanine for measurement of residual white matter and lesion volume. After fixation and cryopreservation in sucrose, 1.5 cm of the cord, encompassing the epicenter was extracted. The cord was transected caudal and rostral with the epicenter in the middle, such that length of each segment is 5 mm. All three 5 mm long pieces were placed rostral to caudal in a square cryostat mold, flush against the right side of the mold and frozen in cryopreservation medium for sectioning. Serial 20  $\mu\text{m}$  coronal sections were collected on 50 sequential Super-Frost slides, resulting in 15 sections per slide (5 per segment). An eriochrome cyanine (EC) staining protocol targeting myelin was developed, and every tenth slide was then stained. The section with the least amount of spared white matter was designated the lesion epicenter.

We have also stained half of the moderately injured mice to analyze serotonergic fiber tracks and developed stereological method of analysis for quantitation of fiber length.

##### **4b. Quantify residual white matter and serotonergic fiber tracks caudal to an injury. (Months 12-32)**

The Eriochrome Cyanine stained spinal cord sections were analyzed in Stereo Investigator using the Cavalieri method. For analysis, a total of 15 sections per animal, at an interval of 200  $\mu\text{m}$  apart were measured. With epicenter section in the center, analysis was performed 1.4 mm in both rostral and caudal directions. The estimated total cord, spared white matter, and lesion volumes were determined. Volumetric analysis for the axial distribution of the lesion and spared white matter across the 3 mm segment was obtained and the percentage of spared white matter and lesion size relative to the total cord were calculated.

We analyzed the axial distribution of the lesion along a 3 mm segment with epicenter in the middle. To account for spinal cord size variability, lesion volume was normalized to cord volume and expressed as percentage lesion volume. Two-way repeated measures ANOVA of percentage lesion volume revealed the following:  $P=0.0714$  for interaction,  $P<0.0001$  for distance, and  $P=0.0001$  for treatment (Figure 20). That is, the size of the lesion decreased at distances further removed from the epicenter in both groups and there was an overall effect of treatment. Sidak's multiple comparisons test revealed significant effect of drug at 400 and 600  $\mu\text{m}$  from epicenter. This shows that drug treated mice had overall smaller lesions as compared to the vehicle treated group.

We next analyzed the axial distribution of spared white matter in the moderately injured mice treated with either GM6001 or vehicle. Two-way repeated measures ANOVA of percentage spared white matter revealed the following:  $P=0.1001$  for interaction,  $P<0.0001$  for distance, and  $P=0.0001$  for treatment (Figure 21). That is, there was greater amount of spared white matter at distances further removed from the epicenter in both groups and there was an overall effect of treatment. Sidak's multiple comparisons test revealed significant effect of drug at 400 and 600  $\mu\text{m}$  from epicenter.

This positive effect of drug on lesion volume and spared white matter was not apparent in the severely injured group (Figures 22 and 23).

**4c. Statistically analyze data. (Months 30-36).**

We have completed statistical analyses of data that were collected.

**REPORT FROM Texas A & M**

***Specific Aims 2-3***

**Task 4. Measure MMPs in CSF in dogs**

**4a. Collect serum from dogs, conduct fluorogenic assays, and analyze data in approximately 125 dogs. (months 12-30)**

Our group entered into a collaboration with Dr. Michael Heller at UC San Diego. Through that work, we have now been able to demonstrate that GM6001 has *in vitro* activity against MMP-2 and MMP-9 at concentrations that approximate those achieved in dog plasma (40-80 ng/mL) 72 hours following a single 100 mg/kg dose subcutaneously. These data together with the complete canine pharmacokinetics support the relevance of this strategy and also suggest that single dosing is likely adequate to achieve reasonably sustained MMP inhibition in dogs. We have already used these assays to analyze canine CSF and serum from dogs with SCI that were administered GM6001 in an NIH funded study. We have now been able to show serum elevation of MMP-2/MMP-9 following SCI and *in vitro* inhibition of serum MMP-2/MMP-9 3 days following delivery of a single 100 mg/kg dose of GM6001. Moreover, this novel assay will serve as a complementary approach to work at UCSF to address MMP activity in CSF using fluorogenic assays.

Additionally, we have begun a collaboration with Mayland Chang at University of Notre Dame to more critically examine metalloproteinase and ADAM activation following SCI. Using banked CSF from dogs with SCI not included in the DoD-funded trial, we have been able to show that the only active MMP detected is MMP-9 and the only active ADAM is ADAM-7 (see appendix).

**BODY**

**Texas A & M Site:**

On 11/6/11 a sub-award agreement between UCSF and Texas A&M University (TAMU) was reached, permitting the ordering of materials to begin work at TAMU. Approvals for key purchases including urodynamics equipment (Laborie Goby), study drug (GM6001, SAI Advantium, India), and pharmacokinetic analysis (KCAS LLC, Kansas, USA) were obtained by mid-December 2011. Specific Aim 2, Task 1 was completed in July 2012, on schedule. Dog enrollment for Specific Aim 2, Task 2 and Specific Aim 3, Task 2 began in November 2012. Dog enrollment for specific Aim 3 task 1 began in July 2012.

Findings, partially supported by this funding, have been submitted for consideration for publication in *Brain*. (Appendix)

***Specific Aim 2***

**Task 1. Study of pharmacokinetics of GM6001 in 10 purpose bred dogs (months 1-12)**

**1a. Obtain animal use protocol approval at Texas A&M University (months 1-4)**

Animal use protocols were approved at TAMU on 8/12/11

### **1b. Order GM6001 drug (months 1-4)**

We were able to obtain permission through our Office of Sponsored Research (OSR) to order GM-6001 in mid-December 2011. A contract was executed with SAI Advantium and processing of the drug began in early January 2012. On March 22 2012, production of 110 g of GM-6001 at HPLC > 98% was completed. The drug was received at TAMU on 4/7/12. Unfortunately, delays associated with obtaining a sub-contract agreement, executing a contract with SAI, and actual drug production resulted in GM 6001 being available in month 8 of the study as opposed to the planned month 4.

### **1c. Order 10 purpose bred dogs (month 4)**

Beagle-like dogs were obtained through the TAMU comparative medicine program in late April 2012, following the availability of GM6001. Dog purchase was delayed as a result of the delays in obtaining GM6001.

### **1d. Receive purpose bred dogs, allow for acclimatization (month 5)**

Dogs were received and acclimatized by early May 2012.

### **1e. Perform physical examination and obtain complete blood count, chemistry, and urinalysis (month 5.5-6)**

### **1f. Anesthetize dogs, place jugular catheters, and deliver GM6001 as a single 100 mg/kg subcutaneous dose (5 dogs) and two 100 mg/kg doses separated by 12 hours (5 dogs) (month 5.5-6)**

(Figure 1, In Supporting Data)

### **1g. Serial serum acquisition (month 5.5-6)**

Objectives 1e-1g were accomplished in mid-May 2012.

### **1h. Samples stored at -80C and shipped to PharmCats for gas chromatography (month 6)**

KCAS was selected as an alternative vendor for pharmacologic studies as they had a lower bid than PharmCats and more rapid turn-around. Samples were shipped to KCAS in mid-May 2012.

### **1i. Samples processed by gas chromatography at PharmCats (months 6-10)**

By mid-June 2012, KCAS generated pharmacokinetic data from dogs. These data were available within the anticipated time frame.

### **1j. Dr. Fajt to analyze pharmacokinetic data (months 10-12). Dr. Fajt will calculate drug elimination half life, peak drug concentration, time to peak concentration, area under the curve, and absorption half life. If serum levels remain elevated beyond the target duration of <5 in the single dose group, drug dose in the IVDH study population will be appropriately adjusted. If serum GM6001 levels are not present for at least 3 days with a single dose protocol, we will consider a 2 dose paradigm in the IVDH study population.**

Dr. Fajt received pharmacokinetic data in mid-June 2012 and completed her analysis by July 1<sup>st</sup> 2012, 2 months ahead of the SOW schedule.

**Summary Task 1:** Delivery of GM6001 was accomplished in 10 purpose bred dogs. All dogs were clinically normal prior to drug administration based on physical examination, neurological examination, complete blood count, serum biochemistry, urinalysis, and CSF analysis. There were few adverse events associated with drug delivery: 10/10 dogs exhibited mild regional hyperesthesia at the delivery site which abated within 1-3 minutes and 10/10 dogs developed transient swelling at the delivery site. Swelling at the delivery site was 2-5 cm in diameter and at the time of the conclusion of the study had decreased in size to 1-3 cm. We have recognized similar swellings in a 4 dog safety study of GM6001 our group previously completed and in 35 dogs that have been administered the drug at 100 mg/kg S.C.

Preliminary analysis of the pharmacokinetics of GM6001 delivered S.C. in dogs suggests a rapid absorption and initial elimination followed by long-elimination half-life ("flip-flop phenomenon"). This pattern required a non-compartmental analysis. GM6001 was detected in plasma at the earliest time point following delivery (5 minutes) and had a mean time to maximal concentration (T<sub>max</sub>) of 0.7 hours (S.D. +/- 1.3 hours). The mean maximal concentration (C<sub>max</sub>) was 1370 ng/mL (S.D. +/- 361 ng/mL). The calculated elimination half-life for a single dose is 524 hours (S.D. +/- 428 hours). The mean concentration of GM6001 following single dose delivery was 80 ng/mL (S.D. +/- 20 ng/mL) at 96 hours.

**Task 2. Compare motor recovery in dogs with IVDH (intervertebral disk herniation) associated SCI that receive saline placebo, DMSO vehicle, or GM 6001 (months 1-36)**

**2a. Obtain animal use protocol approval at Texas A&M University (months 1-4)**

Animal use protocols were approved at TAMU on 8/12/11

**2b. Obtain Clinical Research Review Committee approval (months 1-4)**

Clinical Research Review Committee approval was granted at TAMU on 8/12/11

**2c. Advertise clinical study via electronic brochures (months 6-18)**

In February 2012 UCSF and TAMU began efforts to announce the study to media in order to develop interest in the general public. Stories were featured in the NY Times, ABC News, MS NBC, and on the Today Show website describing this unique collaboration. On June 1 2012 TAMU began efforts to advertise the study to our referring veterinarian population. These efforts included: 1) communications sent to a listserve ("Texasvets") comprised of veterinarians in July 2012, November 2012, February 2013, and June 2013 and 2) mailing electronic PDF brochures to referring veterinarians in November 2012 and March 2013.

**2d. Advertise clinical study via referring veterinarian seminars (months 6-18)**

We have held continuing education events that have featured this study at Veterinary Medical Associations in Montgomery County (September 2012 and February 2013), Washington County (January 2013), and Brazos County (December 2012 and October 2013).

**2e. Advertise clinical study via print media (months 6-18)**

The study was featured in our College's news magazine, "CVM Today" in October 2012

**2f. Development of standardized databases (months 8-10)**

Databases for the study were developed between January and February 2012. Data entry began at the start of enrollment.

**2g. Enrollment of dogs with IVDH (months 13-30)**

Enrollment was initiated in November 2012 (month 15). Currently (month 23), 43/90 dogs have been enrolled. The actual enrollment closely approximates the target of 49 dogs by the close of year 2.

**Summary Task 2:** Just prior to the beginning of enrollment, the study design was altered so that the saline control was eliminated; ACURO was contacted concerning this modification. This was done to enhance our power to detect differences between the DMSO group and GM6001 group. Enrollment has been progressing smoothly, although it appears we are slightly behind in meeting our benchmarks (43 enrollees vs. the 49 anticipated at this time).

**Specific Aim 3**

**Task 1. Compare urodynamic measures in purpose bred dogs and dogs with IVDH (months 1-12)**

**1a. Obtain animal use protocol approval at Texas A&M University (months 1-12)**

Obtained 8/12/2011

**1b. Obtain Clinical Research Review Committee approval (months 1-4)**

Completed 8/12/2011

**1c. Order urodynamic equipment (month 1-4)**

Urodynamic equipment was ordered in mid-December 2011 and arrived at TAMU in February 2011

**1d. Order purpose bred dogs (month 4)**

Purpose bred dogs were ordered in April 2012. As stated previously, this order was delayed due to delays in the production of GM6001.

**1e. Receive purpose bred dogs, allow for acclimatization (month 5)**

Purpose bred dogs were obtained and acclimatized. The acclimatization process was completed in early May 2012.

**1f. Perform urodynamic studies in purpose bred dogs (month 6).**

Ten purpose bred dogs will be utilized. Dogs will be sedated and will have a dual lumen urinary catheter, rectal catheter, and perineal volume following micturition will be recorded and voided volume and voiding efficiency will be calculated. Baseline pressure (vesical pressure after voiding), maximal voiding pressure (maximal vesical pressure during micturition) or leak point pressure (maximal vesical pressure in an animal without voluntary voiding, prior to urine overflow), voiding duration, and voiding interval (the frequency of voiding during filling) will be determined. The number of uninhibited bladder contractions will be recorded on each study. Finally, the timing of external anal sphincter EMG activity in relation to the voiding will be examined. Dogs with phasic contractions of the external anal sphincter during voiding that exhibit subsequent interrupted urine flow and elevated voiding pressure will be classified as having reflex dyssynergia. Voided volume and voiding efficiency will be calculated. Bladder ultrasound will be performed in all dogs immediately following voiding on the same days as urodynamic studies to determine residual urine volume. Animals will be placed in cages and provided water for 8 hours. Upon removal from the cage, dogs will be walked in a large outdoor area and allowed to voluntarily void without manual assistance. Immediately following voiding, an ultrasound machine will be used to measure transverse depth, transverse width, longitudinal length, longitudinal depth, and longitudinal width of the bladder. These measurements will be utilized to calculate residual bladder volume as has been previously described in dogs with IVDH.

Ten healthy beagle-like dogs were utilized to generate experimental data in early May 2012. At the outset of this study, it became clear that Ketamine sedation would be inadequate as it produces excessive spasticity in dogs, which may interfere with the assessment of urodynamic measures. We modified our AUP and received ACURO approval to utilize dexmedetomidine as an alternative sedative agent.

**1g. Perform urodynamic studies in dogs with IVDH (months 6-12). A total of 25 dogs not enrolled in the GM6001 delivery trial will be utilized. Measurements will be performed at admission, and 3 days, 7 days, and 42 days following IVDH surgery. The same cystometric data as outlined in 1f will be recorded.**

On June 1 2012 we opened enrollment to this clinical arm of the study. We have slightly modified inclusion criteria so that dogs lacking deep nociception are excluded due to the severity of the injury. Dogs lacking deep nociception have represented a small fraction (20%) of our IVDH associated SCI caseload and we did not believe that in a 25 dog population of dogs lacking deep nociception to make meaningful conclusions relative to typical urodynamic profile.

To date (9/1/13) we have enrolled 17 of the 25 dogs for this study, 16/17 of which have completed all time points. Data have been exported electronically to Thomas Fandel at UCSF for measurement of critical study variables. Analysis will occur when all animals have been enrolled.

**1h. Dr. Fosgate will analyze data (month 12). Descriptive statistics will be calculated for all urodynamic outcome measures in dogs. Evaluation of descriptive statistics and the Anderson-Darling test will be used to assess the normality assumption. The coefficient of variation will be calculated for the 3 replicates within unaffected dogs to assess the repeatability of the urodynamic measures. Urodynamic measures will be compared between normal and non-trail IVDH dogs at presentation using Student t tests for normally distributed variables and Mann-Whitney U tests otherwise. Outcome measures will be ranked based on the ability to distinguish normal versus affected dogs using scatter plots of standardized values and P values from the statistical comparisons. The outcome measure that best distinguishes affected from normal dogs will be used for subsequent statistical analyses. The most efficient urodynamic measure will also be compared between normal and IVDH-affected dogs at the 42 day recheck evaluation. Repeated measures ANOVA will be used to identify factors associated with improvement of urodynamic measures over time within the IVDH-affected dogs. Predictors that will be evaluated include time from injury until surgery, severity of injury at presentation, surgery duration, and age. Analyses will be performed in commercially available programs and results will be interpreted at the 5% level of significance.**

Formal analysis of these data has not occurred to date as enrollment has not been completed.

**Summary Specific Aim 3, Task 1:** Cystometric measures and post-voiding bladder ultrasound was obtained in 10 Beagle-like dogs with few complications. In 2/10 dogs, hematuria was present following cystometry, but resolved within 24 hours. No dogs developed significant systemic complications as a result of cystometry.

To date, 6 dogs with IVDH-associated SCI have been enrolled in the serial cystometric study. At the time of cystometry, all dogs have been non-ambulatory with 3/6 being paraplegic. Thus, we appear to be obtaining a reasonable balance of mild and moderate SCI dogs. To date, at the time of injury dogs have had absent voiding reflex (5/6), residual volumes that are higher than control dogs, and baseline pressure that is higher than normal. Un-inhibited bladder contractions were seen in 1/6 dogs. Additionally, at the time of SCI bladder capacity appears larger than normal size matched animals. In all dogs, voiding reflexes have been seen by 7 day follow-up cystometry; this has correlated to complete or near-complete bladder emptying recognized on post-voiding ultrasound.

Figures 2 and 3 are cystometrograms obtained at the time of admission and 3 days following admission in one dog that demonstrate patterns seen during injury and recovery. At admission, there was an absence of voiding reflex, leak point pressure of 12 cm H<sub>2</sub>O, 75 mL of residual urine volume, and evidence of un-inhibited bladder contractions (Marked "U"). At 3 days following injury there was an absence of un-inhibited bladder contractions, a maximal voiding pressure of 40 cm H<sub>2</sub>O, voided volume of 78 mL with 28 mL of residual urine, and a baseline pressure following voiding of 8 cm H<sub>2</sub>O.

**Task 2: Compare urodynamic measures in dogs with IVDH enrolled in the GM 6001 delivery trial (months 13-30).**

Enrollment for this arm of the study started in month 15 and to date 43/90 dogs have participated.

## KEY RESEARCH ACCOMPLISHMENTS

### UCSF Site:

- Developed reproducible models of graded SCIs in the mouse.
- Defined key parameters to assess urologic status in mice after SCI
- Conducted the first study to assess efficacy of GM6001 when delivered 8 hours after a moderate SCI injury in mice. Though group sizes were small, these data show promising results in terms of improving neurologic and urologic function.
- Completed all proposed studies in mice to assess efficacy of GM6001 when delivered 8 hours after two levels of injury severities. Here we mirrored the delivery route and vehicle to the dog clinical trial, so drug was dissolved in DMSO and injected subcutaneously.
- We observed therapeutic efficacy of GM6001 on both neurological and urological outcomes in the moderately injured group but not in severely injured group.

### TAMU Site:

- Completed pharmacokinetic study of GM6001 in 10 dogs. The study supports the rapid development of maximal plasma concentration after S.Q. delivery, the presence of plasma drug levels capable of inhibiting MMPs in vitro, and the short term safety of the drug. Plasma drug levels following a single dose are sustained at a significant level likely to result in MMP inhibition for approximately 72 hours, which support a single dose strategy in the clinical trial.
- Completed normal dog cystometry in 10 dogs
- Enrolled 17 dogs with acute IVDH-associated SCI into serial cystometry study (Specific Aim 3.1). As expected, dogs with SCI that are non-ambulatory lack normal voiding reflex, have larger bladder capacity, have elevated post-cystometry baseline pressure, and have larger residual volume compared to measures taken during recovery.
- Enrolled 43 dogs with acute IVDH-associated SCI into clinical trial (Specific Aims 2.2 and 3.2)
- Submitted manuscript to *Brain* (Sept 2013) reporting data from Specific Aim 2.1

## REPORTABLE OUTCOMES

### UCSF Site:

Poster presentation at the MHSRS/ATACC meeting, August 13-16, 2012 in Fort Lauderdale. Abstract was entitled "URINARY BLADDER DYSFUNCTION IN A MURINE MODEL OF SPINAL CORD INJURY: RELATIONSHIP BETWEEN INJURY SEVERITY AND MEASURES OF UROLOGIC STATUS". Abstract is provided in the Appendices.

Invited speaker, International Symposium on Neuroregeneration, December 7, 2011, Asilomar, CA  
MATRIX METALLOPROTEINASES (MMPS) AND SPINAL CORD INJURY

Invited speaker, Ohio State University, January 14, 2013, Columbus, Ohio  
MATRIX METALLOPROTEINASES AS A THERAPEUTIC TARGET FOR SPINAL CORD INJURY

Invited speaker, Rutgers University, W.M. Keck Center for Collaborative Neuroscience January 31, 2013, Piscataway, New Jersey  
MATRIX METALLOPROTEINASES AS A THERAPEUTIC TARGET FOR SPINAL CORD INJURY

Invited speaker, American Veterinary Medical Association, July 19-23, 2013, Chicago, IL  
TARGETING MATRIX METALLOPROTEINASES TO IMPROVE NEUROLOGICAL OUTCOME AFTER SPINAL CORD INJURY: A PATHWAY OF DISCOVERY FROM MICE TO DOGS  
Abstract is provided in the Appendices.

Invited speaker, University of California, September 10, 2013, San Francisco, CA  
TARGETING MATRIX METALLOPROTEINASES TO IMPROVE NEUROLOGICAL OUTCOME AFTER SPINAL CORD INJURY. A PATHWAY OF DISCOVERY FROM MICE TO DOGS

Presenter at nanosymposium, Society for Neuroscience annual meeting, November 9-13, 2013, San Diego, CA  
Abstract was entitled, "ACUTE TREATMENT WITH THE MATRIX METALLOPROTEINASE INHIBITOR GM6001 IMPROVES LONG-TERM LOCOMOTOR AND BLADDER FUNCTION IN A MURINE MODEL OF SPINAL CORD INJURY". Abstract is provided in the Appendices.

Poster presentation at International Symposium on Neuroregeneration, December 11-15, 2013, Asilomar, CA. Abstract was entitled, "MATRIX METALLOPROTEINASES AS A THERAPEUTIC TARGET TO IMPROVE NEUROLOGICAL AND UROLOGICAL FUNCTION AFTER SPINAL CORD INJURY". Abstract is provided in the Appendices.

### TAMU Site:

We have submitted, in collaboration with the UCSF site, a manuscript to *Brain* that details outcomes from our 1<sup>st</sup> canine SCI trial, supported by NIH and data from Specific Aim 2.1 of this study.



## CONCLUSIONS

- In a preliminary study, GM6001 (dissolved in 4% carboxy methyl cellulose and delivered via intraperitoneal route) when given 8 hours after a moderate SCI in the mouse, results in improvement in long-term neurologic recovery and a significant reduction in abnormal bladder contractility.
- Studied efficacy of GM6001 when delivered subcutaneously at 8 hours after moderate and severe SCI in the mouse. GM6001 treatment resulted in a long-term improvement in locomotor function and greater sparing of white matter with a corresponding reduction in lesion volume in the moderate injury group. Awake cystometry revealed reduced residual urine, uninhibited bladder contractions, and bladder wall thickness relative to the vehicle control. In contrast to these beneficial effects of GM6001 seen in the moderate injury group, there were no differences in neurological and urological function between GM6001 and vehicle-treated groups that sustained severe SCIs.
- GM6001 dosed subcutaneously at 100 mg/kg in dogs is safe and results in a pharmacokinetic profile that lends itself to the duration of MMP inhibition demonstrated to be effective in rodent neurotrauma work.
- In dogs with IVDH associated SCI, urinary voiding impairment can be assessed by cystometry and bears similarity to what is seen in humans with per-acute injury. Voiding recovery happens rapidly in dogs with mild or moderate SCI (non-ambulatory with or without limb movement but with intact deep nociception).

**REFERENCES**

1. Noble LJ et al *Matrix metalloproteinases limit functional recovery after spinal cord injury by modulation of early vascular events. Journal of Neuroscience* 22, 7526 (2002)

**APPENDICES:**

Poster presentation at the MHSRS/ATACC meeting, August 13-16, 2012 in Fort Lauderdale.

**TITLE:** Urinary bladder dysfunction in a murine model of spinal cord injury: Relationship between injury severity and measures of urologic status

Thomas M. Fandel, Jonathan Levine, Kayleen Gimlin, Haoqian Zhang,  
Alpa Mahuvakar, and Linda J. Noble-Haeusslein

**Presenter's Name:** Linda J. Noble-Haeusslein, Ph.D.

**PURPOSE/AIMS:** The purpose of this study was to determine the extent to which severity of an incomplete spinal cord injury (SCI) influences bladder function in a murine model of SCI.

**DESIGN:** Mice were randomized to sham, (n=8), mild (n=5) or moderate (n=7) SCI and treated with Enrofloxacin for 10 days subcutaneously followed by food supplemented with Enrofloxacin until euthanasia. Neurological status was evaluated at 1 and 3 days post injury and weekly thereafter for 3 weeks. At 4 weeks post-injury, awake cystometry was performed (n= 3-7/group). At the completion of cystometry and after residual urine was determined (n=3-6/group) the bladders were removed and weighed (n= 5-7/group). All observers were blinded to the experimental condition.

**POPULATION/SAMPLE STUDIED:** Adult, male, C57Bl6 mice subjected to laminectomy only or mild or moderate SCI.

**METHODS:** SCI was produced by dropping either a 2 g (mild injury) or 3 g (moderate injury) weight onto the exposed spinal cord at the T9 vertebral level. Neurological status was based upon the BMS scale. At 3 weeks post-injury, a PE50 polyethylene catheter was implanted into the bladder dome and tunneled subcutaneously to emerge in the interscapular area. One week later, cystometry was performed in the awake restrained animal using saline at an infusion speed of 16-20  $\mu$ l/ minute (Catamount Research, St. Albans, VT). Residual urine was determined at the end of cystometry. The urinary bladders were removed, blotted dry, and weighed.

**DATA ANALYSIS:** Two-way repeated measures (RM) analysis of variance (ANOVA) was used to evaluate neurological recovery. Residual urine and bladder weight were analyzed using 1-way ANOVA followed by Bonferroni's Multiple Comparison Test. Unpaired Student's T-test was used when two groups were specified. Significance was defined at  $P \leq 0.05$ . All data are expressed as means  $\pm$  SEM.

**FINDINGS:** BMS scores revealed an effect of both time ( $p=0.0001$ ) and injury severity ( $p=0.0182$ ). While both injury groups showed improved performance over time, BMS scores were lower in the 3 g ( $1.786 \pm 0.3595$ ) relative to the 2 g ( $6.000 \pm 1.508$ ) group ( $p=0.0097$ ) at 21 days post injury. Moreover, a 3 g injury led to qualitatively more uninhibited bladder contractions and greater residual urine ( $0.9293 \pm 0.1346$ ) and bladder weight ( $0.1475 \pm 0.2238$  g) relative to residual urine ( $0.3980 \pm 0.0080$ ) and bladder weight in the 2 g injury ( $0.07160 \pm 0.0072$  g) ( $p < 0.01$ ).

**CONCLUSIONS/RECOMMENDATIONS:** There are injury severity dependent abnormal changes in both weight and function of the urinary bladder after SCI.

**IMPLICATIONS:** While bladder dysfunction is a common problem in human SCI, analyses of bladder function are typically neglected in murine models of SCI. Characterization of bladder function, relative to injury severity, provides a clinically relevant benchmark for establishing efficacy of candidate therapeutics.

**FROM/TO TIME PERIOD OF STUDY:** From September 30, 2011 to April 25, 2012

**FUNDING:** DOD Spinal Cord Injury Program SC100140

Invited speaker, American Veterinary Medical Association, July 19-23, 2013, Chicago, IL

## TARGETING MATRIX METALLOPROTEINASES TO IMPROVE NEUROLOGICAL OUTCOME AFTER SPINAL CORD INJURY: A PATHWAY OF DISCOVERY FROM MICE TO DOGS

**Presenter's Name:** Linda J. Noble-Haesslein, Ph.D.

Dogs sustain naturally occurring spinal cord injuries (SCIs) represent a clinically relevant population to confirm therapeutic targets that have been identified in rodent models of spinal cord injury (SCI). Spinal cord injured mice, genetically deficient in the gelatinase MMP-9 or treated with GM 6001, a broad-spectrum inhibitor of matrix metalloproteinases (MMPs), beginning 3 hours post injury, show improved long-term neurological outcomes that correspond to the early reduction of leukocytes in the injured cord and stabilization of the blood-spinal cord barrier. Here we find that serum levels of gelatinases are acutely elevated in spinal cord injured dogs, suggesting that these proteases may likewise be a determinant of recovery. After confirming safety and defining the pharmacokinetics of GM 6001 in normal dogs, a large scale, randomized, placebo controlled study was performed in dogs with acute SCIs. Duration of SCI was required to be  $\leq 48$  hours and dogs were stratified according to injury severity. Three groups were studied: GM 6001 + DMSO, DMSO, or saline. As GM 6001 is not soluble in an aqueous solution, DMSO was selected as the vehicle, recognizing that this agent has broad anti-inflammatory actions in models of CNS injury. Only the GM 6001-treated group, given shortly after admission to the clinic, resulted in a reduction in serum gelatinase activity. Utilizing post-hoc statistical techniques, there was a therapeutic benefit of GM 6001+DMSO over DMSO or saline in dogs with mild-moderate spinal cord injuries. These encouraging findings provide the first evidence that MMPs are a determinant of recovery after SCI in dogs. Such validation in a 2nd species reinforces the adverse interactions of these proteases in SCI, and suggests that GM 6001 may likely hold promise for human SCI.

Poster presentation at International Symposium on Neuroregeneration, December 7, 2011, Asilomar, CA.

**MATRIX METALLOPROTEINASES AS A THERAPEUTIC TARGET TO IMPROVE NEUROLOGICAL AND UROLOGICAL FUNCTION AFTER SPINAL CORD INJURY**

A Trivedi, T. M. Fandel, A. F. Martinez, H. Zhang, J. M. Levine, and L. J. Noble-Haeusslein

**Presenter's name:** Alpa Trivedi (Mahuvakar)

We have shown that the matrix-metalloproteinase (MMP) inhibitor, GM6001 improves locomotor function in a murine model of moderate spinal cord contusion injury (SCI), when treatment is initiated at 3 hours post-injury. However, this timing of administration of GM6001 is not easily achievable in the clinical scenario. As infiltrating neutrophils peak at 12 hours post-injury and are a major source of MMPs, we determined if efficacy could be achieved when the timing of administration of GM6001 was extended beyond 3 hours post-injury. In this randomized and blinded study, adult male C57Bl/6 mice were subjected to a moderate-severe or a severe SCI. Animals were randomized to receive drug (GM6001 at 100 mg/kg) or vehicle (99% DMSO) starting 8 hours after injury and then every 12 hours for 3 consecutive days. Using the Basso Mouse Scale (BMS), neurological recovery was assessed at 1 and 3 days post-injury and once per week for 5 weeks. After 5 weeks, awake cystometry was conducted to assess bladder function. GM6001 treatment resulted in a long-term improvement in locomotor function and greater sparing of white matter with a corresponding reduction in lesion volume in the moderate-severe injury group. Awake cystometry revealed reduced residual urine, uninhibited bladder contractions, and bladder wall thickness relative to the vehicle control. In contrast to these beneficial effects of GM6001 seen in the moderate-severe injury group, there were no differences in neurological and urological function between GM6001 and vehicle-treated groups that sustained severe SCIs. These findings demonstrate that GM6001 shows injury-severity dependent efficacy in terms of both neurological and urological recovery. Importantly, this broad-based efficacy is achieved when the drug is administered as late as 8 hours post-injury, a feature which offers promise for the spinal cord injured patient.

## **Efficacy of dimethyl sulfoxide and a metalloproteinase inhibitor in spinal cord injured dogs**

**Authors:** Jonathan M. Levine<sup>1\*</sup>, Noah D. Cohen<sup>2</sup>, Michael Heller<sup>3</sup>, Virginia R. Fajt<sup>4</sup>, Gwendolyn J. Levine<sup>5</sup>, Sharon C. Kerwin<sup>1</sup>, Alpa A. Trivedi, Thomas M. Fandel<sup>6</sup>, Zena Werb<sup>7</sup>, Augusta Modestino<sup>3</sup>, Linda J. Noble-Haeusslein<sup>6,8</sup>

### **Affiliations:**

<sup>1</sup>Department of Small Animal Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, 4474 TAMU, Texas A&M University, College Station, TX 77843 USA.

<sup>2</sup>Department of Large Animal Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, 4474 TAMU, Texas A&M University, College Station, TX 77843 USA.

<sup>3</sup>Department of Bioengineering, University of California-San Diego, San Diego, CA 92093 USA.

<sup>4</sup>Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine and Biomedical Sciences, 4474 TAMU, Texas A&M University, College Station, TX 77843 USA.

<sup>5</sup>Department of Veterinary Pathobiology, College of Veterinary Medicine and Biomedical Sciences, 4474 TAMU, Texas A&M University, College Station, TX 77843 USA.

<sup>6</sup>Department of Neurological Surgery, University of California at San Francisco, San Francisco, CA 94143 USA.

<sup>7</sup>Department of Anatomy, University of California-San Francisco, San Francisco, CA 94143 USA.

<sup>8</sup>Department of Physical Therapy and Rehabilitation, University of California at San Francisco, San Francisco, CA 94143 USA.

\*Address correspondence to: Jonathan M. Levine

Department of Small Animal Clinical Sciences

College of Veterinary Medicine and Biomedical Sciences

4474 TAMU

Texas A&M University

College Station, TX 77843

USA

E-mail: jlevine@cvm.tamu.edu

**Running title:** Acute therapeutics and spinal cord injury

Dogs that sustain naturally-occurring spinal cord injuries represent a clinically-relevant population to confirm therapeutic targets that have been identified in rodent models. Spinal cord-injured mice, deficient in matrix metalloproteinase-9 or treated with GM6001, a broad-spectrum inhibitor of metalloproteinases, show improved long-term neurological outcomes that correspond to the early reduction of leukocytes in the injured cord. Matrix metalloproteinase-9 is abnormally elevated in CSF of dogs sustaining spinal cord injuries, suggesting that matrix metalloproteinases may likewise be determinants of recovery in this species. To test this hypothesis, we confirmed that GM6001 was well tolerated in naïve dogs and described its pharmacokinetics. A single 100 mg/kg subcutaneous dose of GM6001 delivered to naïve dogs resulted in plasma concentrations that peaked shortly after administration and were sustained for at least 4 days at levels that produced robust *in vitro* inhibition of matrix metalloproteinase-9. We then conducted a, randomized, placebo controlled study in dogs to assess efficacy of a single dose of GM6001 and potential effects of its vehicle, dimethyl sulfoxide, given within 48 hours of spinal cord injury. Male and female dogs were enrolled in 3 groups: GM6001 (n=35), dimethyl sulfoxide (n=37), or saline (n=41). Matrix metalloproteinase activity was increased in the serum of spinal cord injured dogs relative to naïve controls. In dogs with spinal cord injury, GM6001 reduced serum matrix metalloproteinase activity compared to dimethyl sulfoxide or saline. To assess recovery, dogs were stratified by a modified Frankel scale into a severely injured group and a mild-to-moderate injured group. The Texas Spinal Cord Injury Score was then used to assess long-term motor/sensory function. All dogs in the mild-to-moderate injury group showed marked recovery with no effect of treatment. However, dogs with severe spinal cord injuries and treated with either GM6001 or dimethyl sulfoxide showed a similar level of long-term improvement that exceeded saline-treated animals. Because GM6001 targets infiltrating neutrophils, we examined a subset of dogs that were treated within 12 hours after spinal cord injury. In this subset, relative to saline, GM6001 improved long-term outcomes in dogs with severe injuries whereas dimethyl sulfoxide treatment did not enhance recovery. Collectively, we demonstrate

efficacy of dimethyl sulfoxide and GM6001, within defined therapeutic windows, in dogs with severe spinal cord injuries. Importantly, our data provide the first evidence that the long-term adverse consequences of severe spinal cord injury in dogs can be modified through acute intervention.

**Key words:** Intervertebral disk herniation, dogs, spinal cord injury, matrix metalloproteinases, neurological recovery, dimethyl sulfoxide

**Abbreviations:** CI = Confidence interval; CSF = Cerebrospinal fluid; CONSORT= Consolidated Standards of Reporting Trials; DMSO= dimethyl sulfoxide; ECM= extracellular matrix; ICCP= International Campaign for Cures of Spinal Cord Injury Paralysis; MMPs= matrix metalloproteinases; MFS= Modified Frankel scale; NINDS= National Institute of Neurological Disorders and Stroke; PK= Pharmacokinetics; SC= Subcutaneously; TSCIS= Texas Spinal Cord Injury Score; T2W= T2-weighted



## Introduction

Matrix metalloproteinases (MMPs) are endopeptidases that degrade the extracellular matrix (Zhang *et al.* , 2010). Several members of the MMP family, including MMP-9 (gelatinase B) and MMP-12, have been implicated in early secondary pathogenesis after spinal cord injury (SCI) (Noble *et al.* , 2002; Wells *et al.* , 2003; Zhang *et al.* , 2011). These MMPs are released by local cells as well as by infiltrating leukocytes and result in reduced cell-cell adhesion, disruption of the blood-spinal cord barrier, up-regulation of pro-inflammatory cytokines, and demyelination (Shigemori *et al.* , 2006; Zhang *et al.* , 2010; Zhang *et al.* , 2011).

Early blockade of MMPs confers neuroprotection after SCI (Lee *et al.* , 2012; Lee *et al.* , 2012; Noble *et al.* , 2002). Short-term administration of the broad spectrum MMP inhibitor, GM6001, results in sparing of white matter and improves locomotor function, when the drug is given over the first 3 days post-injury (Noble *et al.* , 2002). Several lines of evidence suggest that one likely target of GM6001 is MMP-9. This protease is not actively expressed in the uninjured spinal cord and is up-regulated over the first 3 days post-injury, corresponding to the time-course for infiltration of neutrophils (Stirling and Yong, 2008). While there are local sources of MMP-9, including glia and endothelial cells, neutrophil depletion studies confirm that these leukocytes are the major source of MMP-9 in the acutely injured cord (Lee *et al.* , 2012). As this protease is not complexed with tissue inhibitor of matrix metalloproteinase-1, degranulation of neutrophils results in release of activated MMP-9 (Opdenakker *et al.* , 2001), which then may disrupt the barrier and facilitate transmigration of leukocytes into the injured cord. It thus is not surprising that early administration of GM6001 attenuates the trafficking of neutrophils into the injured cord and stabilizes the blood-spinal cord barrier (Noble *et al.* , 2002). There are other members of the MMP family that are also determinants of recovery after SCI including MMP-12 and ADAM-8 (a disintegrin and metalloprotease domain) (Wells *et al.* , 2003). Thus, broad inhibitors of MMPs may offer greater benefit than specific inhibitors of these proteases.

In this study, we have used dimethyl sulfoxide (DMSO) in combination with GM6001 (Kobayashi *et al.* , 2008; Sifringer *et al.* , 2007). While DMSO is commonly used as a vehicle to increase solubility of a drug, others have reported that it is neuroprotective through a presumptive ability to block voltage-sensitive sodium channels and calcium influx into cells, and mitigate opening of ionotropic ion channels that are activated by glutamate (Jacob and de la Torre, 2009).

Few studies have considered a pre-clinical platform involving dogs with naturally occurring SCIs resulting from intervertebral disk herniation (IVDH) (Levine *et al.* , 2011). This approach mimics pathological aspects of human SCI including compressive/contusive injuries and a pro-inflammatory response that includes the infiltration of neutrophils and up-regulation of MMP-9. (Griffiths, 1972; Levine *et al.* , 2006; Smith and Jeffery, 2006). Moreover, these naturally-occurring injuries provide a means for studying therapeutics in the challenging context of varying degrees of injury severity, common in human SCI, but without confounding factors such as anesthetics that are necessary in experimental models.

Here we evaluate the efficacy of GM6001 and DMSO in dogs with IVDH, using an approach that follows recent NINDS recommendations to reduce experimental bias and improve rigor (Landis *et al.* , 2012). Based on a double-blind, randomized, placebo-controlled trial, we show enhanced neurological recovery in dogs sustaining severe SCIs when treated with DMSO within the first 48 hours post-injury. Recovery was likewise improved within a sub-group of dogs with severe SCIs that were admitted within 12 hours of injury and treated with GM6001.

## **Materials and methods**

### **Study Design and Inclusion Criteria**

A preliminary drug tolerance study was constructed based on Food and Drug Administration guidelines (<http://www.fda.gov/AnimalVeterinary/default.htm>) and performed in 4 healthy, purpose-bred Beagles.

Ten healthy, purpose-bred Beagles were obtained to evaluate pharmacokinetics (PK); this sample size was based on similar animal studies and general recommendations for canine PK investigations (Riviere, 1999).

Guidelines for the conduct of SCI trials developed by the International Campaign for Cures of Spinal Cord Injury Paralysis were utilized to assist with the design of a randomized, blinded, placebo-controlled canine trial including inclusion/exclusion criteria, randomization protocol, data handling, and the *a priori* definition of outcome metrics (Lammertse *et al.* , 2007). Consolidated Standards of Reporting Trials (CONSORT) Statement Guidelines were used to assist with trial performance and data reporting (Moher *et al.* , 2010; Schulz *et al.* , 2010). Client-owned dogs with IVDH-associated SCI, admitted to Texas A&M University Veterinary Medical Teaching Hospital between September 2008 and February 2012, were recruited. The study interval was selected to generate a sample size of > 100 dogs, which was considered robust based on previous human phase II and III SCI studies (Dobkin *et al.* , 2007; Geisler *et al.* , 1991), animal model studies of SCI using MMP blockers (Noble *et al.* , 2002), and completed canine SCI studies (Blight *et al.* , 1991; Laverty *et al.* , 2004).

Dogs had to meet the following criteria to be included in the clinical trial population:

1) duration of SCI was required to be  $\leq 48$  hours; 2) IVDH-associated SCI had to result in non-ambulatory paraparesis or paraplegia at enrollment; 3) IVDH-associated SCI had to be identified between the T8-L6 vertebral articulations and treated via surgical decompression. The exclusion criteria were: 1) concurrent disseminated neoplasia or systemic inflammation; 2) a history of recent breeding/pregnancy; and, 3) glucocorticoid treatment within 7 days of SCI.

All animal procedures were approved by the Texas A&M University Institutional Animal Care and Use Committee (AUP 2007-115; AUP 2011-057; AUP-2011-145) and in the case of client-owned dogs were performed with consent. All studies adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

## Drug Preparation, Drug Tolerance, and Pharmacokinetic Procedures

For all canine studies, GM6001 (SAI Advantium, Hyderabad, India) was dissolved in 90% DMSO (Domo, Fort Dodge Corp, Fort Dodge, IA) at a concentration of 250 mg/mL. The solution was sterilized using a 25-mm syringe filter with 0.22- $\mu$ m HT Tuffryn membrane (Pall Corporation, East Hills, NY).

Dogs, participating in the drug tolerance study, were acclimatized for 14 days and then randomized as follows: DMSO, 100 mg/kg GM6001, 150 mg/kg GM6001, or 300 mg/kg GM6001 subcutaneously (SC) every 12 hours for 3 days. The doses of GM6001 were selected to exceed those reported previously in a murine model of SCI (Noble *et al*, 2002). Adverse event monitoring was performed for 7 days following the completion of drug administration. All dogs had physical examinations, injection site evaluations, and assessment of food and water intake twice daily. A complete blood count, serum biochemistry profile, urinalysis, and coagulation profile were performed 3 and 7 days following the completion of drug administration. Following the completion of this study, the vehicle and 300 mg/kg GM6001 dogs were euthanized via intravenous administration of 120 mg/kg pentobarbital (Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI). The brain, heart, liver, kidney, lung, intestine, and injection sites were evaluated.

For PK assessments, a single 100 mg/kg SC administration of GM6001 was delivered in 5 dogs with 5 additional dogs receiving a second 100 mg/kg SC of GM6001, 12 hours following the first dose. In dogs with single dosing, serial plasma samples were obtained at 5, 15, and 30 minutes and 1, 2, 3, 6, 12, 24, 36, 48, and 96 hours after GM6001 delivery. In dogs with multiple dosing, blood samples were collected shortly after the second dose, and then at 24, 48, 72, and 96 hours. All samples were stored in a -80° C freezer until analyzed by high performance chromatography (Thermo Electron Co., Waltham, MA) and tandem mass spectroscopy (MDS-Sciex/Applied Biosystems API3000, Concord, ONT) (LC-MS/MS). GM6001 (m/z 389.0→356.0) concentrations were determined, with MMP Inhibitor III (m/z

364.0→356.0, Calbiochem, Billerica, MA) as the internal standard. A standard curve was created with blank dog plasma at concentrations 10.0 to 10,204.0 ng/mL, with linear regression and weighting of concentrations ( $1/x^2$ ). After thawing and addition of internal standard (300  $\mu$ L of 100 ng/mL in 0.5% acetic acid in methanol), plasma samples or standards (100  $\mu$ L) were centrifuged and reconstituted with 30/70 methanol/10 mM ammonium formate buffer, pH 3.0, for protein precipitation. Supernatant (100  $\mu$ L) was collected, vortexed, and refrigerated until injection on LC-MS/MS. The mobile phase consisted of 0.1% formic acid in deionized water (A) and acetonitrile/methanol/formic acid (40:60:0.1, v/v/v) (B) with a flow rate of 0.30 mL/minute using a linear gradient starting at 40% B from 0 to 0.01 minutes, to 80% B at 1.5 minutes, to 90% B at 3.5 minutes, to 40% B at 3.6 minutes, with a total run time of 5 minutes.

### **Randomized, Placebo Controlled Study in Dogs with IVDH-associated SCI**

Dogs, enrolled in the clinical trial, had physical and neurological examinations, complete blood count and serum biochemistry profile. Anesthesia was induced with propofol (Rapinovel, Schering-Plough Animal Health Corp, Union, NJ) and maintained with inhalant sevoflurane (SevoFlo, Abbott Laboratories, North Chicago, IL). Diagnostic imaging consisting of myelography, computed tomography (CT), or MRI was performed to identify IVDH. Cerebrospinal fluid (CSF) was collected from the cisterna magna for routine analysis and a

200- $\mu$ L aliquot was stored at  $-80^{\circ}$  C for determination of MMP-2/MMP-9 activity. Six mL of whole blood were obtained at the time of CSF collection and 3 days following treatment delivery; serum was isolated and frozen at  $-80^{\circ}$  C.

Immediately after collection of CSF and blood, dogs were randomized to receive 100 mg/kg GM6001+ DMSO, DMSO, or saline placebo in equivalent volumes. A randomization sequence was developed prior to the initiation of this trial and randomization was accomplished by blocking the dogs by

gender status in a 1:1:1 ratio to one of the treatment groups. Sealed envelopes contained treatment allocations and were delivered to a central location where treatments were formulated by individuals not involved in the assessment of animals. Treatments were covered and marked only with animal identifiers to ensure blinding.

Following surgical decompression, all dogs were recovered in an intensive care unit for 24 hours and during that time were provided post-operative opioid analgesia and bladder evacuation. Standard physical rehabilitation protocols consisted of daily pelvic limb passive range of motion and supported standing exercises.

### **Neurological Assessments**

Clinicians responsible for neurologic scoring were blinded to treatment assignments. Two ordinal SCI scores were used to address injury severity at study entry, day 3 post-treatment, and day 42 post-treatment. In both scoring systems, dogs were considered ambulatory if they could spontaneously rise, bear weight, and take at least 10 steps without falling. Dogs that were non-ambulatory had pelvic limb movement evaluated using tail support. Postural responses were evaluated by placing the dorsum of the pes on a non-slick surface while manually supporting the animal and waiting for limb correction. Pelvic limb deep and superficial nociception were evaluated by applying hemostats to a nail-bed or interdigital webbing, respectively and evaluating for the presence of a behavioral or physiological response.

A modified Frankel scale (MFS) was developed to broadly parallel the American Spinal Cord Injury Association Impairment Scale (ASIA) and was subsequently used to stratify the population relative to baseline severity of SCI (Levine *et al.* , 2009; Levine *et al.* , 2011). Dogs were scored as paraplegic with absent deep nociception (0; equivalent to ASIA A), paraplegic with absent superficial nociception (1; equivalent to ASIA B), paraplegic with intact nociception (2; equivalent to ASIA B), or non-ambulatory with identifiable pelvic limb movement (3; equivalent to ASIA C).

The Texas Spinal Cord Injury Score (TSCIS) was used to assess pelvic limb gait, posture and nociception. This is a more refined scale than the MFS (Levine *et al.* , 2011) with a larger array of sub-categories, including gait assessment that parallels the Basso, Beattie, Bresnahan Scale (Basso *et al.* , 1995). The TSCIS gait score ranges from 0 to 6 in each pelvic limb and correlates to the degree of limb protraction and weight bearing. The gait classifications include: no voluntary movement seen when the dog is supported (score = 0), intact limb protraction with no ground clearance (1), intact limb protraction with inconsistent ground clearance (2), intact protraction with ground clearance > 75% of steps (3), ambulatory with consistent ground clearance and significant paresis-ataxia that results in occasional falling (4), ambulatory with consistent ground clearance and mild paresis-ataxia that does not result in falling (5), and normal gait (6). Pelvic limb postural responses using the TSCIS were scored in each limb as absent (0), delayed (1, correction occurred >1 second after positioning), and present (2). Nociception was scored in each limb as absent (0), deep nociception only present (1), or deep and superficial nociception both present (2).

### **Magnetic Resonance Imaging (MRI)**

Vertebral column MRI was performed on enrolled dogs, except in cases where animals were evaluated outside of normal operating hours or the scanner was unavailable due to mechanical failure. Between September 2008 and July 2011, a 1.0 T system (Siemens Magnetom, Malvern, PA) was utilized to acquire images; for the remainder of the trial images were generated using a 3.0 T MRI (Siemens Verio, Malvern, PA). Dogs that received MRI had sagittal T2-weighted (T2W) images reviewed by 1 investigator (JML) using commercially available software (eFILM, Merge Healthcare, Chicago, IL) prior to un-blinding. The acquisition parameters for sagittal T2W images generated at 1T and 3T included a repetition time of 3500 ms, echo time of 90 ms, and slice thickness of 2.0 mm. The presence of spinal cord T2W hyperintensity visualized on sagittal images was recorded.

## MMP-2/MMP-9 Activity in CSF and Serum

CSF and serum samples (n= 16/ treatment group) were randomly selected at the end of the trial by computerized sorting on random numbers. Purpose-bred Beagle dogs (n= 5) were sampled as controls. Serum samples with overt hemolysis were excluded from analysis.

Activity of MMP-2 and MMP-9 in serum and CSF samples was assessed blinded using a previously developed electrophoretic method (Lefkowitz *et al.* , 2010; Lefkowitz *et al.* , 2010; Lefkowitz *et al.* , 2010) that included a synthetic peptide (AAPptec, Louisville, KY) (sequence: Ac-NGDPVGLTAGAGK-NH<sub>2</sub>), tagged with a fluorophore BODIPY-FL-SE (Invitrogen, Carlsbad, CA). The substrate was mixed with either serum or CSF, with phosphate buffered saline as the negative control. After reacting for 1 hour, aliquots were loaded onto 20% polyacrylamide gels and the samples were electrophoresed. Gels were imaged using a BioDoc-It M-26 transilluminator (UVP, Upland, CA, USA). The image was scanned in a Storm 840 workstation (Molecular Dynamics, Sunnyvale, CA, USA) with ImageQuant v5.2 software and fluorescent signal was quantified using ImageJ (1.440, National Institutes of Health, Bethesda, MD).

To assess ability of GM6001 to inhibit MMP-9, activity (described above) was determined using human recombinant MMP-9 (Sigma, St. Louis, MO) that was diluted in DMSO to final concentrations of 0.01  $\mu$ M to 200  $\mu$ M. Controls consisted of enzyme and substrate only and substrate and GM6001 only.

## Statistical Analyses

Noncompartmental pharmacokinetic analysis was performed (Phoenix WinNonLin 6.3, Pharsight, St. Louis, MO), and estimates of the parameters of  $T_{\max}$ ,  $C_{\max}$ ,  $T_{1/2}$ , and area  $AUC_{0-\text{obs}}$  and  $AUC_{0-\infty}$  were calculated for the single dose.

Activities of MMP-2/MMP-9 in CSF and serum were compared between healthy control dogs and the dogs with SCI using Student's t-test. The Wilcoxon rank-sum test was used to compare CSF and



serum MMP-2/MMP-9 activities between dogs with and without selected characteristics that were potential modifiers of MMP-2/MMP-9 (i.e., age, breed, sex, and markers of disease duration or severity). To compare serum MMP-2/MMP-9 values among treatment groups, the serum MMP-2/MMP-9 activities were converted to ranks, and the ranks were compared using a generalized linear model; post-hoc pairwise comparisons between treatments were made using the method of Sidak. Model fit was assessed graphically using diagnostic plots of residuals.

For the clinical trial data, baseline characteristics were compared among the 3 treatment groups to determine whether there was any evidence of differences among groups. Categorical variables were compared using chi-squared analysis and continuous or ordinal variables were compared using Kruskal-Wallis tests. The primary outcome for the trial was the TSCIS score on day 42. The total TSCIS on day 3 was considered a secondary outcome. The association of TSCIS with treatment group and other individual variables was assessed using generalized linear modeling. Individual variables significantly associated with TSCIS were analyzed using multivariable generalized linear modeling using maximum likelihood estimating methods. Post-hoc testing was performed using the method of Sidak. Model fit was assessed graphically using diagnostic plots of residuals. Comparisons of proportions among treatment groups (e.g., frequency of adverse events) were made using chi-squared or, when appropriate, Fisher's exact tests. Significance was set at  $P < 0.05$  for all analyses. Analyses were performed using S-PLUS statistical software (Version 8.2, TIBCO, Inc., Seattle, WA).

## Results

### GM6001 is well tolerated in naive dogs

We first addressed the safety of GM6001 using a dose tolerance study. Four uninjured dogs were randomized to receive DMSO vehicle, 100 mg/kg GM6001, 150 mg/kg GM6001, or 300 mg/kg GM6001 SC every 12 hours for 3 days. Following drug delivery, all studied parameters were within normal limits, with the following exceptions: 1) increase in body temperature in all GM6001-treated dogs which peaked 6 days after treatment was completed (Supplementary Fig. S1); 2) transient decrease in food consumption during the 3 days of drug delivery (mean percentage of food consumed,  $64.5\% \pm 12.9\%$ ) in comparison to the 3 days following delivery (mean percentage of food consumed,  $91.7\% \pm 16.3\%$ ); and 3) the presence of subcutaneous nodules at the drug delivery sites that regressed in size following delivery (Supplementary Fig. S2). No lesions were detected via necropsy or histopathology in the dog that received vehicle. In the dog receiving 300 mg/kg GM6001 twice daily for 3 days, sites of subcutaneous drug deposition were surrounded by a connective tissue capsule with minimal inflammation; additionally, there was mild bile duct hyperplasia. The absence of substantial adverse events in this tolerance study suggested that GM6001 would have an acceptable safety profile in injured dogs.

### GM6001 is rapidly detected in plasma after subcutaneous administration

As the PK of GM6001 may differ from that in rodent (Mahmood, 2010), we determined the PK in normal dogs. GM6001, administered once at 100 mg/kg SC, was detected in plasma at 5 minutes in all dogs, with a mean time to peak concentration ( $T_{max}$ ) of 0.7 hours (S.D.  $\pm 1.3$  hours) (Fig. 1). The mean peak concentration ( $C_{max}$ ) was 1370 ng/mL (S.D.  $\pm 361$  ng/mL), mean apparent elimination half-life ( $T_{1/2}$ ) was 524 hours (S.D.  $\pm 428$  hours), and the mean plasma concentration of GM6001 at 96 hours was 80 ng/mL (S.D.  $\pm 20$  ng/mL). Mean area under the curve from time zero to last observed concentration ( $AUC_{0-obs}$ ) ( $16,100 \text{ hr} \cdot \text{ng/mL} \pm 2981$ ) and mean area under the curve from time zero to infinity ( $AUC_{0-\infty}$ ) ( $58,225$

hr\*ng/mL  $\pm$  37,054) result in an extrapolated percentage of AUC of 65%. The GM6001 utilized in the clinical trial had marked *in vitro* MMP-9 inhibition at concentrations approximating those achieved in dog plasma 96 hours post-drug delivery (Fig. 2). As the objective was to target the acutely injured cord, we selected a single 100 mg/kg SC dose of GM6001 in dogs to achieve plasma drug concentrations which would peak almost immediately after delivery and be sustained at levels sufficient to inhibit MMPs *in vitro* for at least 96 hours following delivery.

### **Clinical Trial Enrollment**

Enrolled dogs were randomized to a saline placebo group (n= 38 dogs), a DMSO group (n= 36), and a GM6001 group (n= 33) (Fig. 3). Three dogs were euthanized prior to discharge from the hospital due to neurologic deterioration and 16 dogs did not return for 42-day follow-up examination.

### **Adverse Events in Spinal Cord Injured Dogs**

Adverse events were recorded during hospitalization and were classified as fever, gastrointestinal, injection site, urinary, or other (Supplementary Table S1). A significantly greater number of dogs in the GM6001 group had injection site reactions (45%; 15/33) relative to either the saline control dogs (5%; 2/38) or DMSO dogs (0%; 0/36) ( $P < 0.0001$ ; Kruskal-Wallis test). These reactions were transient and consisted of focal dermal and subcutaneous swelling.

### **Increased MRI T2W signal within the spinal cord is associated with recovery**

Vertebral column MRI was performed on 76/107 dogs enrolled in the clinical trial. In all cases, spinal cord compression associated with IVDH was identified with variable presence of increased T2W signal (27/76 dogs) within the spinal cord (Figure 4). High spinal cord T2 signal,

suggestive of contusion, was significantly more common in dogs with severe SCIs (MFS=0; 11/13) compared with those with mild-moderate SCIs (MFS>0; 16/63) ( $P=0.0001$ ; Fisher's exact test). Dogs with increased T2W spinal cord also had significantly ( $P < 0.0001$ ; generalized linear model) poorer recovery of function 42 days following SCI (estimated TSCIS 9, 95% CI 7-12) compared to dogs with normal spinal cord T2W signal (estimated TSCIS 15, 95% CI 14-17). The presence of compressive SCI with variable presence of T2W hyperintensity in the spinal cord parallels what is found on MRI in humans with traumatic myelopathy, including relationships between function and spinal cord T2 signal (Miyajima *et al.*, 2007).

### **Characterization of cells in CSF following spinal cord injury**

CSF was acquired immediately prior to drug or placebo delivery in 102/107 (95%) clinical trial dogs; 5/5 un-injured control animals also had CSF collected. Total nucleated cell count was significantly ( $P=0.0034$ , Wilcoxon rank-sum test) higher in SCI dogs (median = 3 cells/ $\mu$ L, range 0-71) compared with control dogs (median = 0 cells/ $\mu$ L, range 0-1). Amongst dogs with CSF pleocytosis (total nucleated cell count > 5 cells/ $\mu$ L), neutrophils were most frequently identified (median 43%, range 2-89%), followed by mononuclear cells (median 25%, range 6-95%) and lymphocytes (median 18%, range 4-70%). CSF red blood cell count was likewise significantly ( $P=0.0022$ , Wilcoxon rank-sum test) increased in dogs with SCI (median 48 cells/ $\mu$ L, range 0-15,040). Together, these findings support a pro-inflammatory state in the acutely injured canine spinal cord.

### **GM6001 reduces gelatinase activity in serum of spinal cord injured dogs**

We utilized a fluorescent electrophoretic technique to determine if MMP-2/MMP-9 activity increases in CSF and serum in dogs with SCI and whether activity is reduced after treatment (Lefkowitz *et al.* , 2010; Lefkowitz *et al.* , 2010; Lefkowitz *et al.* , 2010). MMP-2/MMP-9 activity in the CSF did not differ between dogs with SCI and control dogs ( $P=0.3841$ ; Student's t-test) (Supplementary Table S2, Supplementary Fig. S3). Dogs with SCI had significantly ( $P = 0.0086$ ; Student's t-test) higher serum MMP-2/MMP-9 activity prior to treatment compared with control dogs, but activity did not vary based on clinical factors or MRI features of SCI (Table 1, Fig. 5). Serum MMP-2/MMP-9 activity was significantly ( $P<0.05$ ; generalized linear model) lower in dogs receiving GM6001 3 days following treatment compared to dogs receiving either DMSO or saline (Fig. 6). Thus, these findings confirm the effectiveness of GM6001 in reducing MMP-2/9 in serum.

### **DMSO enhances recovery in dogs with severe spinal cord injuries**

Dogs were stratified into those with behaviorally severe SCI (MFS = 0; absent pelvic limb movement and deep nociception) and those with mild-moderate SCI (MFS >0; intact pelvic limb deep nociception with or without movement) to examine whether treatment group affected TSCIS at 3- and 42-day time-points. This stratification scheme was utilized because a substantially lower proportion of dogs with severe SCI recover independent ambulation at long-term follow-up time-points (approximately 50-60%) in comparison to dogs with mild-to-moderate SCI (approximately 85 to 95%); thus, injury severity might influence the ability to detect treatment-related effects (Ferreira *et al.* , 2002; Ito *et al.* , 2005; Olby *et al.* , 2003; Ruddle *et al.* , 2006)]. Critically, there were no differences in baseline population characteristics such as breed, gender, or injury level among treatment groups, suggesting that confounding based on these parameters was unlikely (Table 2).

In dogs with mild-to-moderate SCI (i.e., MFS >0), there was robust recovery of function by 42 days with 64/71 (90%) of dogs independently walking and 69/71 having intact pelvic limb nociception. Treatment group did not influence 3 or 42 day TSCIS (Fig. 7, Supplementary Fig. S4). Dogs with mild-to-moderate SCI had significantly higher 42-day TSCIS (mean 15; 95% CI 12-18) compared to those with severe SCI (mean 7; 95% CI 4-9) ( $P < 0.0010$ ; generalized linear model). In dogs with severe SCI (i.e., MFS = 0), those receiving either DMSO or GM6001 had significantly ( $P < 0.05$ ; generalized linear model with Sidak post-hoc comparisons) more robust functional recovery compared with those receiving saline placebo (Fig. 7) at 42 days. Sub-components of the 42-day TSCIS were examined in dogs with severe SCI to better capture the influence of treatment on motor, sensory, and postural recovery. Using generalized linear modeling, dogs receiving saline had an estimated mean motor score of 2 (95% CI 0-4.0) (suggesting absent to minimal pelvic limb movement with tail support) that was significantly ( $P < 0.05$ ; generalized linear model with Sidak post-hoc comparisons) less than the estimated mean motor score for dogs receiving DMSO (mean, 5; 95% CI 2.0-8.0) or GM6001 (mean, 5; 95% CI 2.0-8.0). The distribution of motor scores for both the DMSO and GM6001 treated dogs indicated that the majority of animals in these groups developed coordinated stepping movements with tail support. In dogs with severe SCI, the majority that recovered pelvic limb movements (11/13; 85%) also regained nociception; treatment group was not significantly associated with postural or nociceptive scores. Importantly, the extent of neurological recovery at 42 days post-injury was similar in DMSO- and GM6001 (dissolved in DMSO)-treated groups.

### **Neurological recovery is improved in a sub-group of dogs treated with GM6001**

The neuroprotective effects mediated by GM6001 in rodents with SCI most likely rely on early, rapid blockade of MMPs that arise from infiltrating neutrophils (Lee *et al.*, 2012). Thus, we hypothesized that GM6001 might act in a time-dependent manner in dogs with SCI. To test this hypothesis, we stratified

the population by duration of injury at the time of enrollment ( $\leq 12$  hours and  $>12$  hours) and examined 42-day TSCIS. A 12-hour cut-point was selected as neutrophil infiltration into the spinal cord following injury has been shown to peak during this time-frame (Stirling and Yong, 2008). There were no differences in baseline population characteristics among treatment groups in dogs enrolled  $\leq 12$  hours or  $>12$  hours after spinal cord injury (Supplementary Tables S3 and S4).

In dogs with injury duration of  $\leq 12$  hours and severe SCI (i.e., MFS = 0), GM6001 treatment (n=4) was associated with significant ( $P = 0.0086$ ; generalized linear model) improvement at 42 days compared to saline control (n=4) based upon the TSCIS (Fig. 8). DMSO treatment (n=3) did not result in recovery that was significantly different than saline control. Using generalized linear modeling, dogs receiving GM6001 had an estimated 42-day motor score of 7 (95% CI 4-11) whereas those receiving saline had a significantly lower estimated 42-day motor score of 2 (95% CI 0-5) ( $P=0.0069$ ). Nociceptive and postural scores did not differ significantly between treatment groups. Treatment group did not affect 42-day TSCIS in dogs with mild-to-moderate SCI (i.e., MFS  $> 0$ ) enrolled  $\leq 12$  hours after SCI and did not impact recovery in dogs enrolled  $>12$  hours after SCI. A post-hoc analysis was performed restricting analysis to dogs examined within 24 hours of injury to further explore the therapeutic window of DMSO and GM6001. Findings paralleled those seen for the overall population of dogs enrolled (data not shown); namely, both DMSO and GM6001 resulted in significantly improved 42-day TSCIS compared to saline in dogs with severe SCI. In summary, in dogs with severe SCI enrolled within 12 hours of injury, only GM6001 exerts a positive effect on TSCIS 42 day outcome relative to saline control.

## Discussion

This study was designed as a large-scale clinical trial to evaluate MMP inhibition and DMSO in a clinically-relevant, naturally-occurring canine SCI model. Here we show improved neurological function

in dogs with severe SCIs that were treated with DMSO within 48 hours of injury. At 42 days post-injury, these dogs showed robust stepping movements that were visible with tail support; saline-treated dogs either showed no movement or had minimal limb advancement without stepping. Additionally, we demonstrate a therapeutic effect of GM6001, a broad-spectrum MMP inhibitor, in a sub-group of dogs with severe SCIs that were administered treatment within 12 hours of injury. While GM6001 significantly reduced MMP-2/MMP-9 in serum of SCI dogs, DMSO had no effect on these serum proteases. Such findings suggest that the benefit seen in DMSO-treated dogs was independent of an ability to modulate MMP activity.

DMSO is perhaps best known for its ability to dissolve both polar and nonpolar molecules (Jacob and de la Torre, 2009). As such it is commonly used as a vehicle for delivery of drugs that exhibit low solubility in aqueous solution. However, there is evidence that DMSO, when used alone, may confer neuroprotection to the central nervous system. Based upon earlier studies, DMSO is thought to function as a free radical scavenger and suppress a variety of pathobiologic events including inflammation, calcium influx, and glutamate excitotoxicity in experimental models of brain and spinal cord injury (Jacob and de la Torre, 2009). Such broad-based, temporally-defined targets may account for the extended window of efficacy (within 48 hours of injury) in spinal cord-injured dogs. Most recently, the efficacy of an epidermal growth factor receptor inhibitor dissolved in DMSO was assessed in a rodent model of SCI (Sharp *et al.*, 2012). Recovery of motor and bladder function was significantly improved in rats that received DMSO relative to the inhibitor. Despite the absence of a saline control, we suggest that DMSO did not serve as the “baseline” but rather may have functioned as a neuroprotectant.

There are several additional factors that make DMSO a candidate for human trials. DMSO is currently approved by the Federal Drug Administration for the treatment of human interstitial cystitis (Parkin *et al.*, 1997). Thus, there is opportunity to repurpose DMSO. Moreover, as DMSO has an



extended therapeutic window (48 hours), time to treatment, which may be delayed for up to 1 to 3 days post-injury (Furlan *et al.* , 2012), is feasible.

DMSO, when co-administered with a second candidate therapeutic, offers potential for synergism, by acting through separate and/or overlapping pathways. Such synergism, for example, has been reported in a model of brain ischemia where DMSO was combined with fructose 1,6-disphosphate, an intermediate of anaerobic metabolism, and prostacyclin (PG<sub>12</sub>) which blocks aggregation of platelets and functions as a vasodilator (de la Torre, 1991; de la Torre, 1995). We considered the possibility that DMSO in combination with GM6001 would be synergistic in supporting recovery. However, neurological recovery was no different in those dogs that were treated with both drugs, relative to DMSO alone.

While we found that DMSO improved neurologic function in those dogs that sustained severe SCIs, a similar improvement was not seen after mild-moderate SCIs. The lack of treatment-related effects in this latter group may be related to their pronounced long-term recovery (64/71 [90%] independently walked at 42 days) that reduced the likelihood of detecting a treatment effect. Others have similarly shown that 85% to 95% of mildly to moderately injured dogs with IVDH recover independent ambulation (Aikawa *et al.* , 2012; Ruddle *et al.* , 2006). The high proportion of animals recovering ambulation with mild-to-moderate SCI is parallel to what is seen in humans with incomplete motor lesions (ASIA C and D) where 76% to 100% of patients achieve functional recovery (Al-Habib *et al.* , 2011; Geisler *et al.* , 2001).

Although our findings are strongly supportive of DMSO as a safe therapeutic for dogs with severe SCI, untoward systemic side-effects, including bronchospasm, hypertension, and renal failure have been reported by others (Santos *et al.* , 2003) and published findings of efficacy remain equivocal. We found no significant adverse effects of DMSO in this study, perhaps because the treatment consisted of a single subcutaneous dose. The fact that DMSO is not widely accepted as a therapeutic may be in part related to the lack of replication of findings. For example, while DMSO has been reported to be neuroprotective in a

model of traumatic brain injury (Di Giorgio *et al.* , 2008), others have found no beneficial effect on measures of behavioral outcomes after traumatic brain injury. The challenge is comparing studies as experimental designs are often markedly different. In addition, beneficial effects of DMSO often occur serendipitously, when it is used as a vehicle and no additional control is included in the experiment.

GM6001 is a broad-spectrum MMP inhibitor that has been shown to exert neuroprotection in rodent models of brain and spinal cord injuries, primarily via antagonism of MMP-9 (Zhang *et al.* , 2010). Evidence supporting this position includes a temporal association between neutrophil trafficking and MMP-9 expression, reduced expression of MMP-9 in spinal cord-injured mice that are neutrophil-depleted, and reduced neutrophil content within injured spinal cords of MMP-9 null mice (Noble *et al.* , 2002; Stirling and Yong, 2008).

This strong association between MMP-9 and neutrophils suggests that an optimal therapeutic window for GM6001 is defined by the early trafficking of neutrophils into the injured cord. Such a position is supported by evidence of pronounced neurological recovery when the drug was given beginning 3 hours after post-injury in a murine model of SCI (Noble *et al.* , 2002). In the current study, neurological improvement was only seen in a subset of dogs that were treated with GM6001 within 12 hours of injury. While the population of severe SCI dogs treated within 12 hours (n=11) is small, the findings do suggest that the therapeutic window for GM6001 is defined by adverse events that occur in the acute period post-injury.

The lack of a robust response associated with GM6001 may have also been due to prolonged activity of this drug. Pharmacokinetics in healthy dogs demonstrated the plasma concentration of GM6001, present at even the 96-hour time-point, approximated or exceeded that necessary to block MMP-9 *in vitro*. As some MMPs modulate the formation of a glial scar and axonal plasticity, (Zhang *et al.* , 2011), their subacute/chronic blockade may result in adverse neurological outcomes.

The experimental design of this study employed early stratification according to injury severity. We included dogs with a spectrum of severities, a feature common to human clinical trials. We recognize that when outcome effects are limited to a particular sub-group, confounding is more likely. We attempted to mitigate this possibility by performing comparisons between injury strata relative to baseline dog characteristics; no inter-group differences were identified. A single outcome measure, the TSCIS, was used to evaluate long-term neurologic recovery. We selected this scale as it has excellent inter-rater agreement, correlates to MRI features of SCI, and predicts long-term functional outcome in IVDH SCI (Levine *et al.* , 2009). While ordinal systems analogous to the TSCIS are a well-accepted means to evaluate outcomes in human SCI trials, they have drawbacks including potential limited sensitivity to detect subtle changes in gait or posture and the non-continuous nature of the data (Lammertse, 2013). As such multiple tests, including kinematic assessments, would have added more detail to our neurological assessments. Despite such limitations, the experimental design was sufficient to define a therapeutic, perhaps in part because the supportive data were so robust.

In summary, while there have been a number of therapeutics that have enhanced recovery in rodent models of SCI, they have failed to produce similar benefit when evaluated in large-scale human clinical trials (Casha *et al.* , 2012; Geisler *et al.* , 2001; Lammertse *et al.* , 2012). Two critical factors implicated in poor translation are failure to validate in a second species (Kwon *et al.* , 2010; Levine *et al.* , 2011) and lack of rigor in experimental design (Lammertse, 2013; Steward *et al.* , 2012). Using a trial design that included randomization, blinding, and previously validated functional outcomes, we identify DMSO as a candidate therapeutic for human SCI. Additionally, the administration of GM6001 results in MMP blockade, which is neuroprotective only if initiated within 12 hours of SCI..

## Acknowledgements:

We thank Ms. Alisha Selix for coordinating canine trial enrollment and providing technical support for canine studies. We also thank Dr. George Lemieux for advice on the inhibitor.

## Funding:

This study was supported by funds from NIH R01NS039278 (Supp) and DoD SC100140. Dr. Cohen was supported in part by the Link Equine Research Endowment.

## References

- Aikawa T, Fujita H, Kanazono S, Shibata M, Yoshigae Y. Long-term neurologic outcome of hemilaminectomy and disk fenestration for treatment of dogs with thoracolumbar intervertebral disk herniation: 831 cases (2000-2007). *Journal of the American Veterinary Medical Association* 2012; 241: 1617-26.
- Al-Habib AF, Attabib N, Ball J, Bajammal S, Casha S, Hurlbert RJ. Clinical predictors of recovery after blunt spinal cord trauma: systematic review. *Journal of Neurotrauma* 2011; 28: 1431-43.
- Basso DM, Beattie MS, Bresnahan JC. A sensitive and reliable locomotor rating scale for open field testing in rats. *J Neurotrauma* 1995; 12: 1-21.
- Blight AR, Toombs JP, Bauer MS, Widmer WR. The effects of 4-aminopyridine on neurological deficits in chronic cases of traumatic spinal cord injury in dogs: a phase I clinical trial. *J Neurotrauma* 1991; 8: 103-19.
- Casha S, Zygun D, McGowan MD, Bains I, Yong VW, Hurlbert RJ. Results of a phase II placebo-controlled randomized trial of minocycline in acute spinal cord injury. *Brain : a journal of neurology* 2012; 135: 1224-36.
- de la Torre JC. Synergic activity of combined prostacyclin: dimethyl sulfoxide in experimental brain ischemia. *Can J Physiol Pharmacol* 1991; 69: 191-8.
- de la Torre JC. Treatment of head injury in mice, using a fructose 1,6-diphosphate and dimethyl sulfoxide combination. *Neurosurgery* 1995; 37: 273-9.
- Di Giorgio AM, Hou Y, Zhao X, Zhang B, Lyeth BG, Russell MJ. Dimethyl sulfoxide provides neuroprotection in a traumatic brain injury model. *Restor Neurol Neurosci* 2008; 26: 501-7.
- Dobkin B, Barbeau H, Deforge D, Ditunno J, Elashoff R, Apple D, et al. The evolution of walking-related outcomes over the first 12 weeks of rehabilitation for incomplete traumatic spinal cord injury: the multicenter randomized Spinal Cord Injury Locomotor Trial. *Neurorehabil Neural Repair* 2007; 21: 25-35.
- Ferreira AJ, Correia JH, Jaggy A. Thoracolumbar disc disease in 71 paraplegic dogs: influence of rate of onset and duration of clinical signs on treatment results. *J Small Anim Pract* 2002; 43: 158-63.
- Furlan JC, Tung K, Fehlings M. Process Benchmarking Appraisal of Early Surgical Decompression of Spinal Cord following Traumatic Cervical Spinal Cord Injury: Opportunities to Enhance the Time to Definitive Treatment. *Journal of Neurotrauma* 2012.
- Geisler FH, Coleman WP, Grieco G, Poonian D, Group SS. Measurements and recovery patterns in a multicenter study of acute spinal cord injury. *Spine* 2001; 24S: S68-S86.
- Geisler FH, Dorsey FC, Coleman WP. Recovery of motor function after spinal-cord injury--a randomized, placebo-controlled trial with GM-1 ganglioside. *The New England journal of medicine* 1991; 324: 1829-38.
- Griffiths IR. Some aspects of the pathology and pathogenesis of the myelopathy caused by disc protrusions in dogs. *J Neurol Neurosurg Psychiatry* 1972; 35: 403-13.
- Ito D, Matsunaga S, Jeffery ND, Sasaki N, Nishimura R, Mochizuki M, et al. Prognostic value of magnetic resonance imaging in dogs with paraplegia caused by thoracolumbar intervertebral disk extrusion: 77 cases (2000-2003). *J Am Vet Med Assoc* 2005; 227: 1454-60.
- Jacob SW, de la Torre JC. Pharmacology of dimethyl sulfoxide in cardiac and CNS damage. *Pharmacol Rep* 2009; 61: 225-35.
- Kobayashi H, Chattopadhyay S, Kato K, Dolkas J, Kikuchi S, Myers RR, et al. MMPs initiate Schwann cell-mediated MBP degradation and mechanical nociception after nerve damage. *Mol Cell Neurosci* 2008; 39: 619-27.

- Kwon BK, Hillyer J, Tetzlaff W. Translational research in spinal cord injury: a survey of opinion from the SCI community. *J Neurotrauma* 2010; 27: 21-33.
- Lammertse D, Tuszynski MH, Steeves JD, Curt A, Fawcett JW, Rask C, et al. Guidelines for the conduct of clinical trials for spinal cord injury as developed by the ICCP panel: clinical trial design. *Spinal Cord* 2007; 45: 232-42.
- Lammertse DP. Clinical trials in spinal cord injury: lessons learned on the path to translation. The 2011 International Spinal Cord Society Sir Ludwig Guttmann Lecture. *Spinal Cord* 2013; 51: 2-9.
- Lammertse DP, Jones LA, Charlifue SB, Kirshblum SC, Apple DF, Ragnarsson KT, et al. Autologous incubated macrophage therapy in acute, complete spinal cord injury: results of the phase 2 randomized controlled multicenter trial. *Spinal Cord* 2012; 50: 661-71.
- Landis SC, Amara SG, Asadullah K, Austin CP, Blumenstein R, Bradley EW, et al. A call for transparent reporting to optimize the predictive value of preclinical research. *Nature* 2012; 490: 187-91.
- Lavery PH, Leskova A, Breur GJ, Coates JR, Bergman RL, Widmer WR, et al. A preliminary study of intravenous surfactants in paraplegic dogs: polymer therapy in canine clinical SCI. *J Neurotrauma* 2004; 21: 1767-77.
- Lee JY, Kim HS, Choi HY, Oh TH, Ju BG, Yune TY. Valproic acid attenuates blood-spinal cord barrier disruption by inhibiting matrix metalloproteinase-9 activity and improves functional recovery after spinal cord injury. *Journal of neurochemistry* 2012; 121: 818-29.
- Lee JY, Kim HS, Choi HY, Oh TH, Yune TY. Fluoxetine inhibits matrix metalloproteinase activation and prevents disruption of blood-spinal cord barrier after spinal cord injury. *Brain : a journal of neurology* 2012; 135: 2375-89.
- Lefkowitz RB, Marciniak JY, Hu CM, Schmid-Schonbein GW, Heller MJ. An electrophoretic method for the detection of chymotrypsin and trypsin activity directly in whole blood. *Electrophoresis* 2010; 31: 403-10.
- Lefkowitz RB, Schmid-Schonbein GW, Heller MJ. Whole blood assay for elastase, chymotrypsin, matrix metalloproteinase-2, and matrix metalloproteinase-9 activity. *Analytical chemistry* 2010; 82: 8251-8.
- Lefkowitz RB, Schmid-Schonbein GW, Heller MJ. Whole blood assay for trypsin activity using polyanionic focusing gel electrophoresis. *Electrophoresis* 2010; 31: 2442-51.
- Levine GJ, Levine JM, Budke CM, Kerwin SC, Au J, Vinayak A, et al. Description and repeatability of a newly developed spinal cord injury scale for dogs. *Prev Vet Med* 2009; 89: 121-7.
- Levine JM, Levine GJ, Porter BF, Topp K, Noble-Haeusslein LJ. Naturally occurring disk herniation in dogs: an opportunity for pre-clinical spinal cord injury research. *J Neurotrauma* 2011; 28: 675-88.
- Levine JM, Ruaux CG, Bergman RL, Coates JR, Steiner JM, Williams DA. Matrix metalloproteinase-9 activity in the cerebrospinal fluid and serum of dogs with acute spinal cord trauma from intervertebral disk disease. *Am J Vet Res* 2006; 67: 283-7.
- Mahmood I. Theoretical versus empirical allometry: Facts behind theories and application to pharmacokinetics. *J Pharm Sci* 2010; 99: 2927-33.
- Miyanji F, Furlan JC, Aarabi B, Arnold PM, Fehlings MG. Acute cervical traumatic spinal cord injury: MR imaging findings correlated with neurologic outcome - prospective study with 100 consecutive patients. *Radiology* 2007; 243: 820-7.
- Moher D, Hopewell S, Schulz KF, Montori V, Gøtzsche PC, Devereaux PJ, et al. CONSORT 2010 explanation and elaboration: updated guidelines for reporting parallel group randomised trials. *Bmj* 2010; 340: c869.
- Noble LJ, Donovan F, Igarashi T, Goussev S, Werb Z. Matrix metalloproteinases limit functional recovery after spinal cord injury by modulation of early vascular events. *J Neurosci* 2002; 22: 7526-35.
- Olby N, Levine J, Harris T, Munana K, Skeen T, Sharp N. Long-term functional outcome of dogs with severe injuries of the thoracolumbar spinal cord: 87 cases (1996-2001). *J Am Vet Med Assoc* 2003; 222: 762-9.
- Opdenakker G, Van den Steen PE, Dubois B, Nelissen I, Van Coillie E, Masure S, et al. Gelatinase B functions as regulator and effector in leukocyte biology. *J Leukoc Biol* 2001; 69: 851-9.
- Parkin J, Shea C, Sant GR. Intravesical dimethyl sulfoxide (DMSO) for interstitial cystitis--a practical approach. *Urology* 1997; 49: 105-7.
- Riviere JE. Study Design and Data Analysis. *Comparative Pharmacokinetics: Principles, Techniques, and Applications*. Ames, Iowa: Iowa State University Press; 1999. p. 239-58.
- Ruddle TL, Allen DA, Schertel ER, Barnhart MD, Wilson ER, Lineberger JA, et al. Outcome and prognostic factors in non-ambulatory Hansen type I intervertebral disc extrusions: 308 cases. *Vet Comp Orthop Traumatol* 2006; 19: 29-34.
- Santos NC, Figueira-Coelho J, Martins-Silva J, Saldanha C. Multidisciplinary utilization of dimethyl sulfoxide: pharmacological, cellular, and molecular aspects. *Biochem Pharmacol* 2003; 65: 1035-41.
- Schulz KF, Altman DG, Moher D. CONSORT 2010 statement: updated guidelines for reporting parallel group randomised trials. *Bmj* 2010; 340: c332.

- Sharp K, Yee KM, Steward O. A re-assessment of the effects of treatment with an epidermal growth factor receptor (EGFR) inhibitor on recovery of bladder and locomotor function following thoracic spinal cord injury in rats. *Experimental neurology* 2012; 233: 649-59.
- Shigemori Y, Katayama Y, Mori T, Maeda T, Kawamata T. Matrix metalloproteinase-9 is associated with blood-brain barrier opening and brain edema formation after cortical contusion in rats. *Acta Neurochir Suppl* 2006; 96: 130-3.
- Sifringer M, Stefovská V, Zentner I, Hansen B, Stepulak A, Knaute C, et al. The role of matrix metalloproteinases in infant traumatic brain injury. *Neurobiology of disease* 2007; 25: 526-35.
- Smith PM, Jeffery ND. Histological and ultrastructural analysis of white matter damage after naturally-occurring spinal cord injury. *Brain Pathol* 2006; 16: 99-109.
- Steward O, Popovich PG, Dietrich WD, Kleitman N. Replication and reproducibility in spinal cord injury research. *Experimental neurology* 2012; 233: 597-605.
- Stirling DP, Yong VW. Dynamics of the inflammatory response after murine spinal cord injury revealed by flow cytometry. *Journal of neuroscience research* 2008; 86: 1944-58.
- Wells JE, Rice TK, Nuttall RK, Edwards DR, Zekki H, Rivest S, et al. An adverse role for matrix metalloproteinase 12 after spinal cord injury in mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2003; 23: 10107-15.
- Zhang H, Adwanikar H, Werb Z, Noble-Haeusslein LJ. Matrix metalloproteinases and neurotrauma: evolving roles in injury and reparative processes. *Neuroscientist* 2010; 16: 156-70.
- Zhang H, Trivedi A, Lee JU, Lohela M, Lee SM, Fandel TM, et al. Matrix metalloproteinase-9 and stromal cell-derived factor-1 act synergistically to support migration of blood-borne monocytes into the injured spinal cord. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2011; 31: 15894-903.

Table 1: Values of serum MMP 2/9 activity were not significantly associated with various clinical variables. Medians (and ranges) and P values derived from Wilcoxon-rank sum tests are reported for the categorical variables listed below.

<b>Variable</b>	<b>Median (Range) of MMP 2/9</b>	<b>Activity in Serum</b>	<b>P value</b>
<b>Age</b>	<b><math>\leq 5</math> Years (<math>N = 28</math>)</b> 1,519,998 (1,009,205 – 3,708,449)	<b><math>&gt; 5</math> Years (<math>N = 12</math>)</b> 1,315,180 (769,191 – 2,700,981)	0.1458
<b>Sex</b>	<b>Male (<math>N = 22</math>)</b> 1,504,968 (1,009,205 – 3,708,449)	<b>Female (<math>N = 18</math>)</b> 1,455,587 (769,191 – 2,700,981)	0.2625
<b>Neutered</b>	<b>Not neutered (<math>N = 9</math>)</b> 1,492,947 (1,009,205 – 2,266,638)	<b>Neutered (<math>N = 31</math>)</b> 1,479,017 (769,191 – 3,708,449)	0.5881
<b>Breed</b>	<b>Dachshund (<math>N = 28</math>)</b> 1,411,546 (769,191 – 3,144,395)	<b>Other (<math>N = 12</math>)</b> 1,528,755 (941,766 – 3,708,449)	0.4932
	<b>Chondrodysplastic (<math>N = 38</math>)</b> 1,474,618 (769,191 – 3,708,449)	<b>Other (<math>N = 2</math>)</b> 1,983,362 (1,975,379 – 1,991,345)	0.2564
<b>Duration of clinical signs prior to admission</b>	<b><math>\leq 12</math> hours (<math>N = 12</math>)</b> 1,453,961 (1,009,205 – 3,708,449)	<b><math>&gt; 12</math> hours (<math>N = 28</math>)</b> 1,485,982 (769,191 – 3,144,395)	0.9189
	<b><math>\leq 24</math> hours (<math>N = 35</math>)</b> 1,492,947 (941,766 – 3,708,449)	<b><math>&gt; 24</math> hours (<math>N = 5</math>)</b> 1,310,144 (769,191 – 2,306,259)	0.5243
<b>T2-weighted hyperintensity</b>	<b>Absent (<math>N = 20</math>)</b> 1,507,977 (769,191 – 3,708,449)	<b>Present (<math>N = 11</math>)</b> 1,523,790 (1,062,500 – 3,144,395)	0.6995
<b>MFS score at admission</b>	<b><math>\leq 2</math> (<math>N = 22</math>)</b> 1,455,587 (1,009,205 – 2,292,539)	<b><math>&gt; 2</math> (<math>N = 18</math>)</b> 1,504,968 (769,191 – 3,708,449)	0.4924

Table 2: Baseline characteristics did not differ significantly among treatment groups. Panel a. summarizes continuous variables using medians (ranges) by group, with P values from Kruskal-Wallis tests; panel b describes categorical variables using proportions by group with P values from chi-squared testing. \* MFS = Modified Frankel scale; # TSCIS = Texas Spinal Cord Injury score

**Change a. Continuous variables: Medians (range); P value from Kruskal-Wallis testing**

<b>Variable</b>	<b>Saline Controls (N = 38)</b>	<b>DMSO (N = 36)</b>	<b>Drug+DMSO (N = 33)</b>	<b>P</b>
<b>Age (years)</b>	5 (2 to 13)	5 (3 to 13)	5 (2 to 14)	0.9833
<b>Duration of signs prior to admission (hours)</b>	24 (1 to 48)	18 (4 to 36)	12 (2 to 48)	0.2246
<b>MFS*</b>	2 (0 to 3)	2 (0 to 3)	2 (0 to 3)	0.7409
<b>TSCIS#</b>	4 (0 to 10)	4 (0 to 11)	4 (0 to 10)	0.5907

**Change b. Categorical variables: P values from chi-squared testing**

<b>Variable</b>	<b>Saline Controls (N = 38)</b>	<b>DMSO (N = 36)</b>	<b>Drug+DMSO (N = 33)</b>	<b>P</b>
<b>Sex</b>				
<b>Female</b>	61% (23/38)	53% (19/36)	39% (13/33)	0.3039
<b>Male</b>	39% (15/38)	47% (17/36)	61% (20/33)	
<b>Neutered</b>				
<b>No</b>	16% (6/38)	22% (8/36)	18% (6/33)	0.9078
<b>Yes</b>	84% (32/38)	78% (28/36)	82% (27/33)	
<b>Breed</b>				
<b>Dachshund</b>	71% (27/35)	61% (22/36)	85% (28/33)	0.1560
<b>Other</b>	29% (8/35)	39% (14/36)	15% (5/33)	
<b>Chondrodys-</b>				
<b>trophoid</b>	89% (34/38)	86% (31/36)	88% (29/33)	0.9628
<b>Other</b>	11% (4/38)	14% (5/36)	12% (4/33)	
<b>Level of Spinal Cord Injury</b>				
<b>T12-T13</b>	34% (13/38)	36% (13/36)	24% (8/33)	0.6986
<b>T13-L1</b>	29% (11/38)	25% (9/36)	33% (11/33)	0.8705
<b>L1-L2, L2-L3, 29% (11/38)</b>		33% (12/36)	24% (8/33)	0.8390
<b>or L3-L4</b>				
<b>T2-Weighted Hyperintensity (only available for 76 dogs)</b>				
<b>Absent</b>	62% (18/29)	76% (22/29)	50% (9/18)	0.3225



Present	38% (11/29)	24% (7/29)	50% (9/18)
---------	-------------	------------	------------

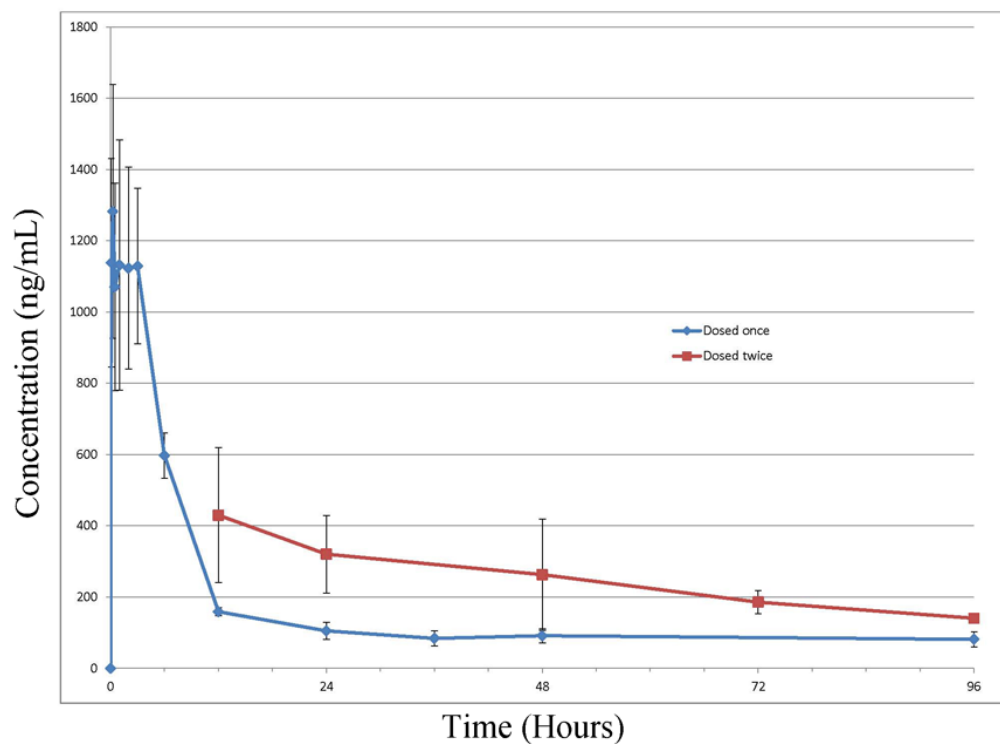


Figure 1: Administration of a single 100 mg/kg subcutaneous dose of GM6001 to dogs resulted in the rapid development of peak plasma drug concentrations with drug still detectable 96 hours post-delivery. Administration of a second dose of GM6001 to a sub-group of dogs 12 hours following initial drug delivery resulted in increased plasma drug concentrations at all assessed time points.

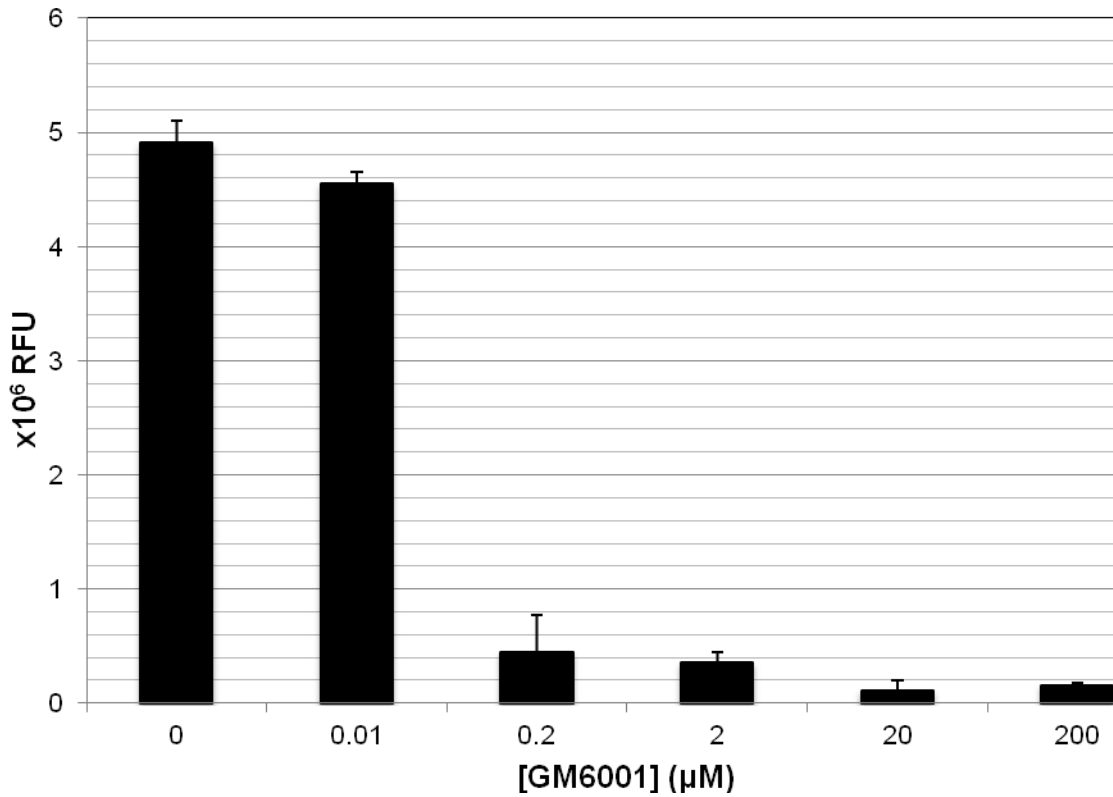
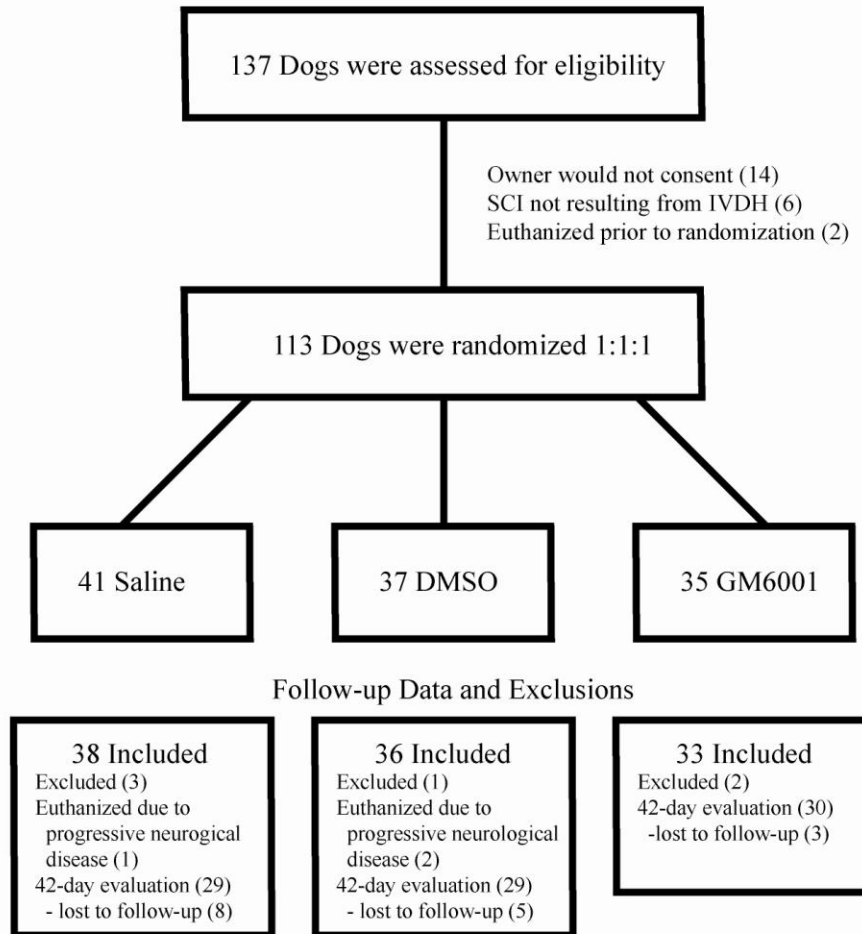


Figure 2: Calibrated MMP-9 activity (Log 10<sup>6</sup>), as measured by a fluorescent electrophoretic technique, was dramatically attenuated by GM6001 *in vitro* at various concentrations. Plasma concentrations of GM6001, measured 96 hours following a single 100 mg/kg subcutaneous dose (range 0.16-.26 μM or 60-100 ng/mL), approximated those needed *in vitro* to robustly inhibit MMP-9 (0.2μM or 77 ng/mL).

Figure 3. Flow diagram depicting progress through different phases of the clinical trial including enrollment, group allocation, and follow-up



\* Dogs were excluded from data analysis due to the fact that they were ambulatory when randomized, which was not permitted according to inclusion criteria (n= 4) or due to herniation of an adjacent disk during the course of the trial (n=1) .

Figure 4: Sagittal T2-weighted magnetic resonance images from 2 dogs with spinal cord injuries resulting from intervertebral disk herniation that led to compressive and contusive primary injury. In 1 dog (A) that was non-ambulatory with intact pelvic limb movement and sensation, there was focal spinal cord compression at the T12-T13 vertebral articulation without spinal cord signal change. A second dog (B) with paraplegia and absent pelvic limb deep nociception had compression at the T12-T13 vertebral articulation and extensive spinal cord T2-weighted hyperintensity (white arrows), suggestive of processes seen in contusion injuries such as edema, necrosis, hemorrhage, or cellular infiltrates.

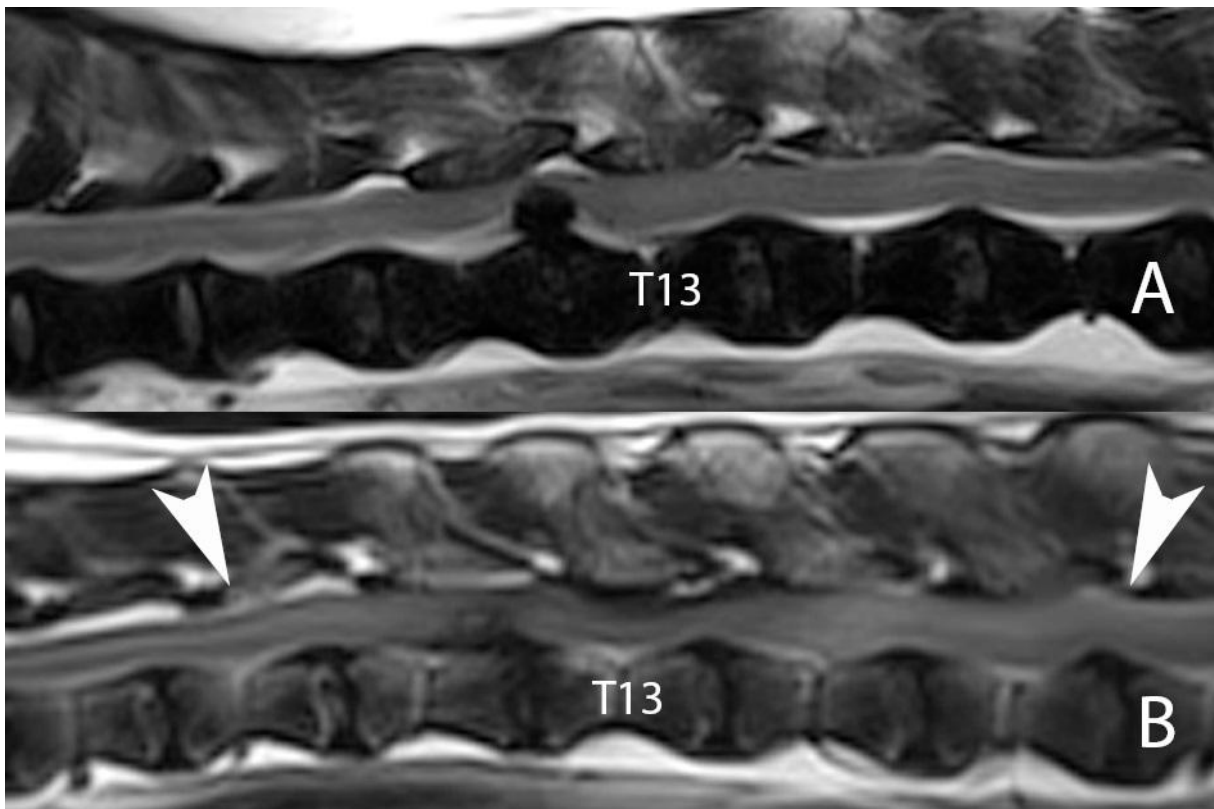


Figure 5: Values of serum MMP 2/9 activities were significantly ( $P = 0.0086$ ; t-test) greater for dogs with spinal cord injury (SCI;  $N = 40$ ) included in the trial than control dogs ( $N = 5$ ). Bar graph summarizing the mean serum MMP 2/9 activity of healthy dogs ( $N = 5$ ) and dogs with spinal cord injuries (SCI) that had CSF collected and tested ( $N = 40$ ). Bars represent mean values; error bars represent the standard deviation.

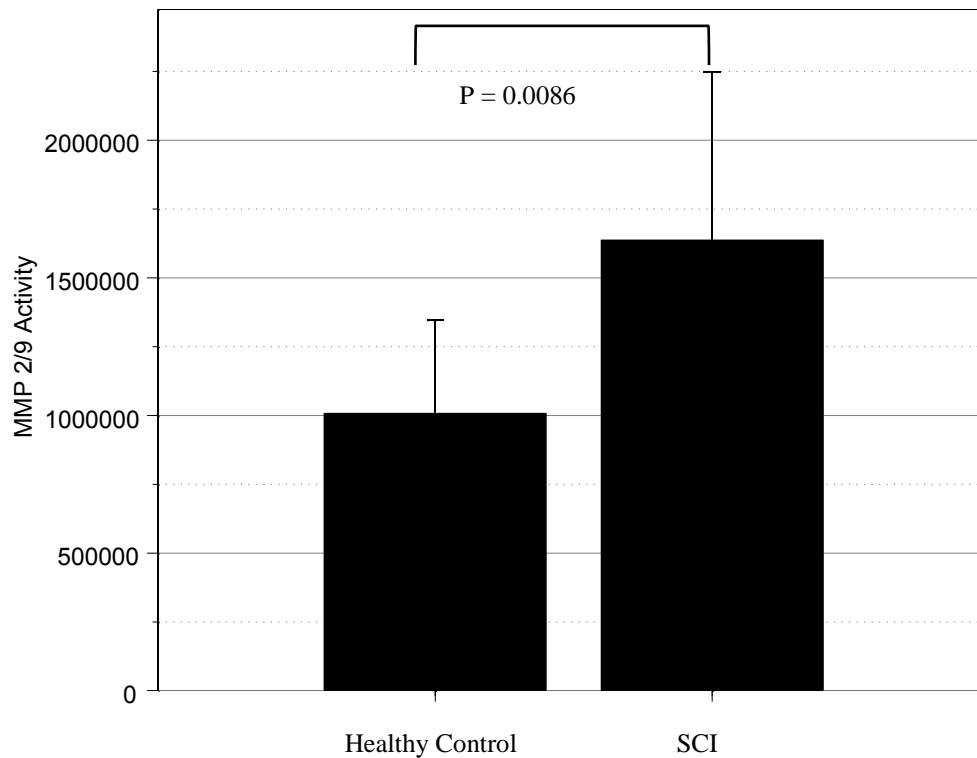


Figure 6: Dogs were first randomized into 3 treatment groups, and serum was obtained prior to administration of saline, DMSO, or GM6001 (panel a). There were no differences in serum MMP-2/MMP-9 activity among treatment groups at the time of admission (panel a); however, at day 3, there was a significant ( $P < 0.05$ ) difference in serum activity between GM6001-treated dogs and the other treatment groups (panel b). Bars represent mean values; error bars represent the standard deviation. Groups marked with different letters differ significantly ( $P < 0.05$ )

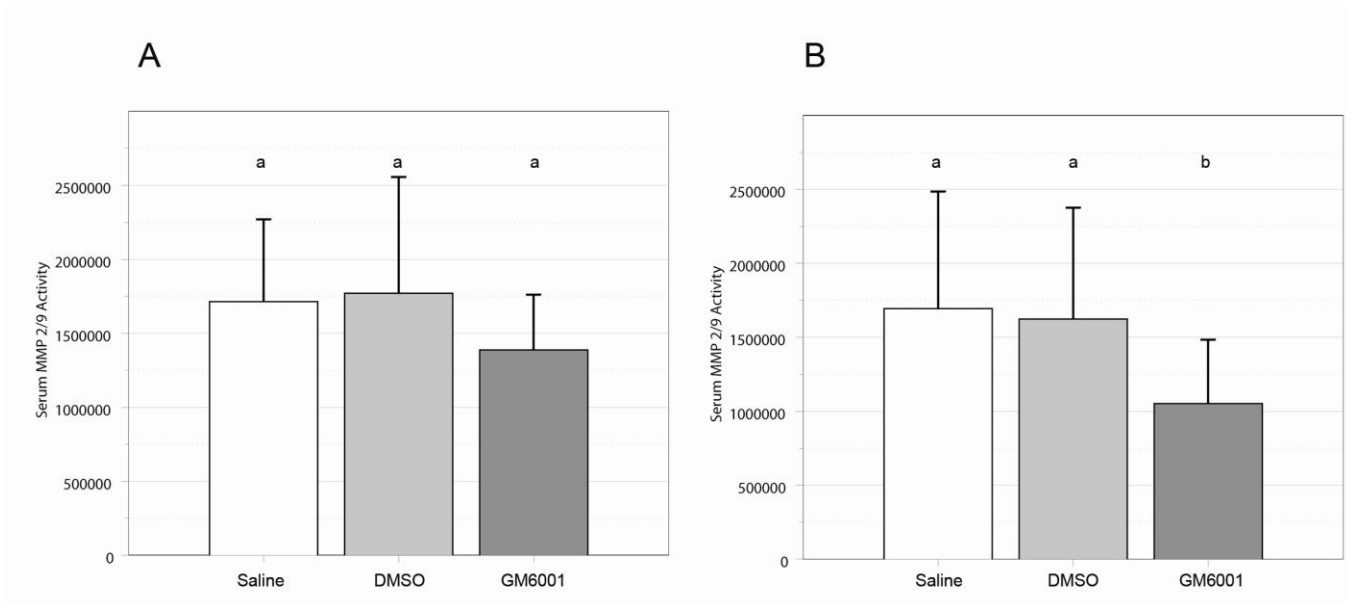


Figure 7: Bar graph of mean TSCIS scores on day 42 by treatment group, stratified by MFS score at admission (MFS = 0, left panel; or MFS > 0, right panel). The error bars represent the upper bound of the symmetrical 95% confidence intervals. The letters along the top of the panels represent significant ( $P < 0.05$ ) differences after adjusting for multiple comparisons: treatment groups with different letters differed significantly ( $P < 0.05$ ) following multiple comparisons. There were no significant differences in TSCIS among dogs with MFS score > 0 (right panel), but TSCIS was significantly ( $P < 0.05$ ) greater for the GM6001 group and the DMSO group than saline group among dogs with MFS = 0 (left panel).

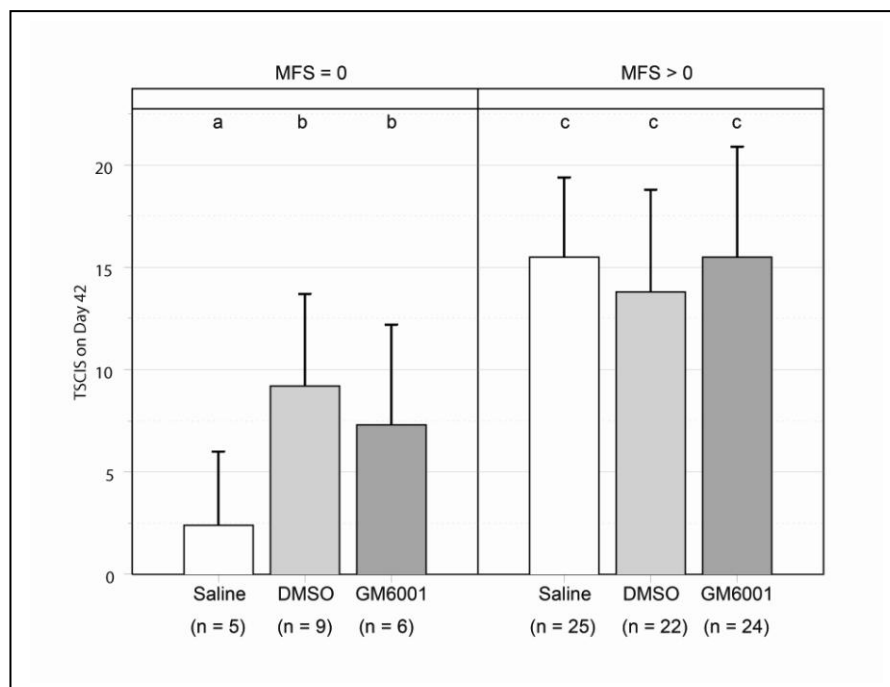
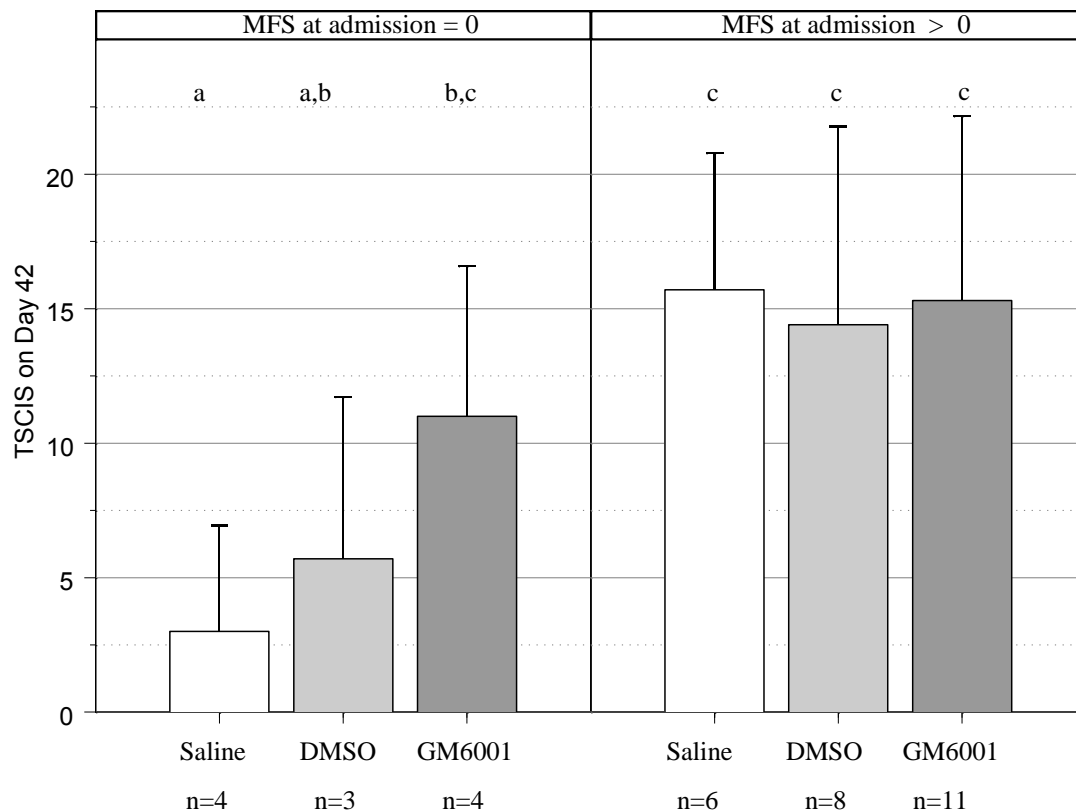
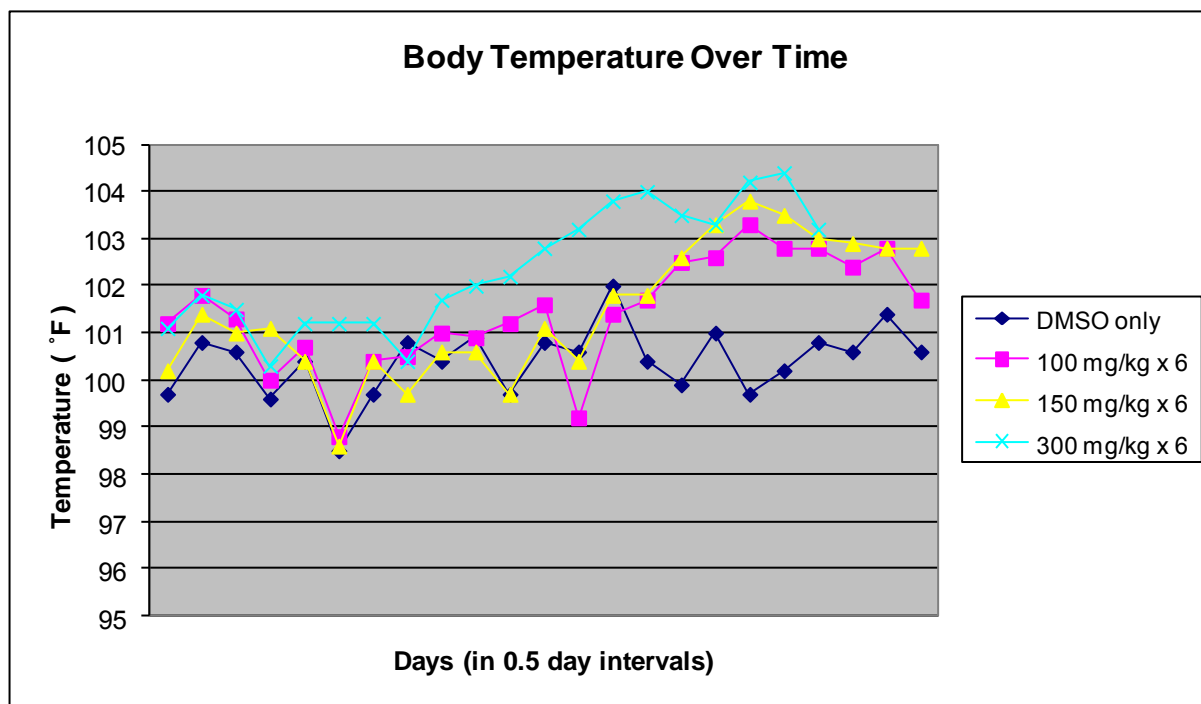


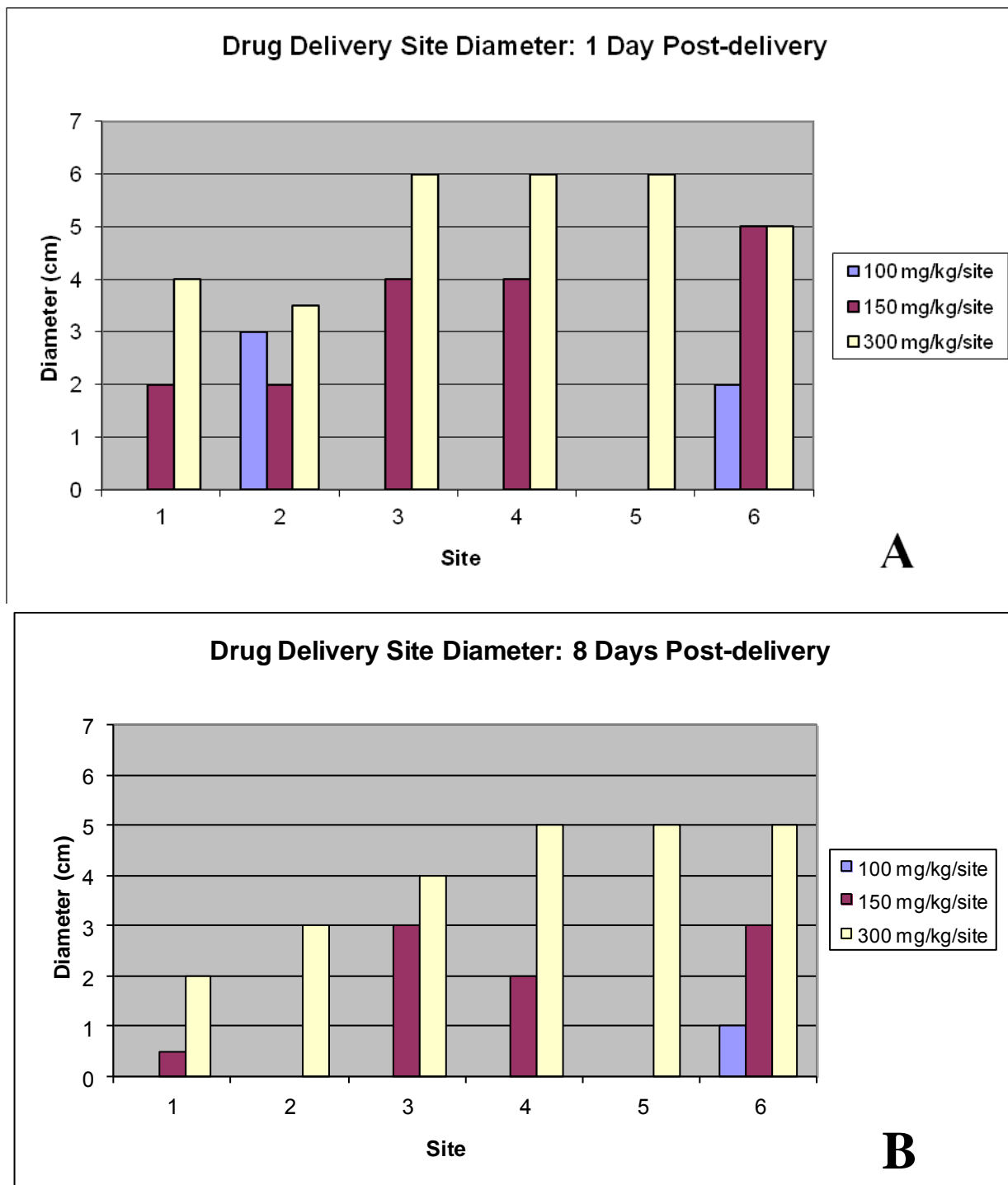


Figure 8: Bar graph of mean TSICS scores at day 42 among dogs with spinal cord injury by treatment, stratified by MFS score at admission (left panel, MFS score = 0 at admission; right panel, MFS score > 0 at admission). The error bars represent the upper bound of the symmetrical 95% confidence intervals. The letters along the top of the panels represent significant ( $P < 0.05$ ) differences after adjusting for multiple comparisons: treatment groups with different letters differed significantly ( $P < 0.05$ ) following multiple comparisons. The TSCIS scores for dogs treated with GM6001 were significantly ( $P = 0.0086$ ) greater than those of saline controls in the MFS=0 strata. For saline controls and DMSO-treated dogs, TSCIS scores were significantly ( $P < 0.05$ ) higher for dogs with MFS > 0 at admission than for dogs with MFS = 0; however, among dogs treated with GM6001 there was no significant difference in the TSCIS values between dogs with MFS = 0 and MFS > 0 at admission.



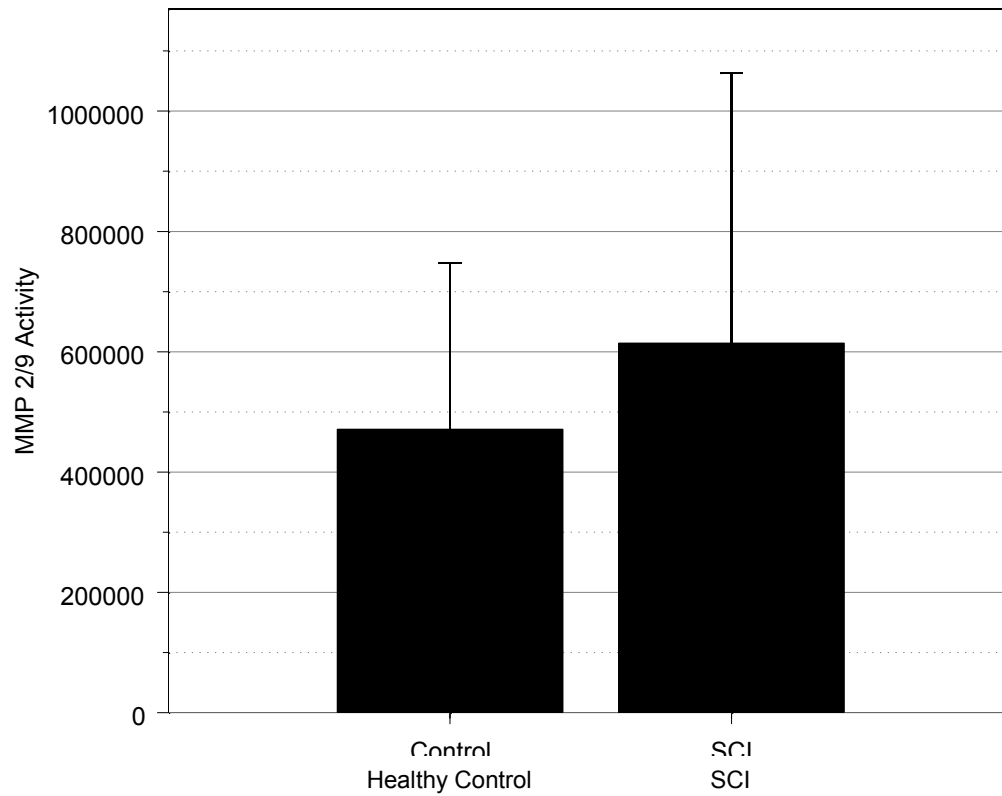


Supplemental Figure 1: Rectal body temperature recorded over time in dogs delivered DMSO or GM6001 at 6-18 times the cumulative clinical trial dose. All dogs delivered GM6001 experienced body temperature elevations beyond what is normal for a dog ( $>102.5^{\circ}$  F) whereas the dog that was delivered DMSO remained normal throughout the study. The elevation in body temperature qualitatively appeared greatest in dogs receiving higher doses of GM6001.

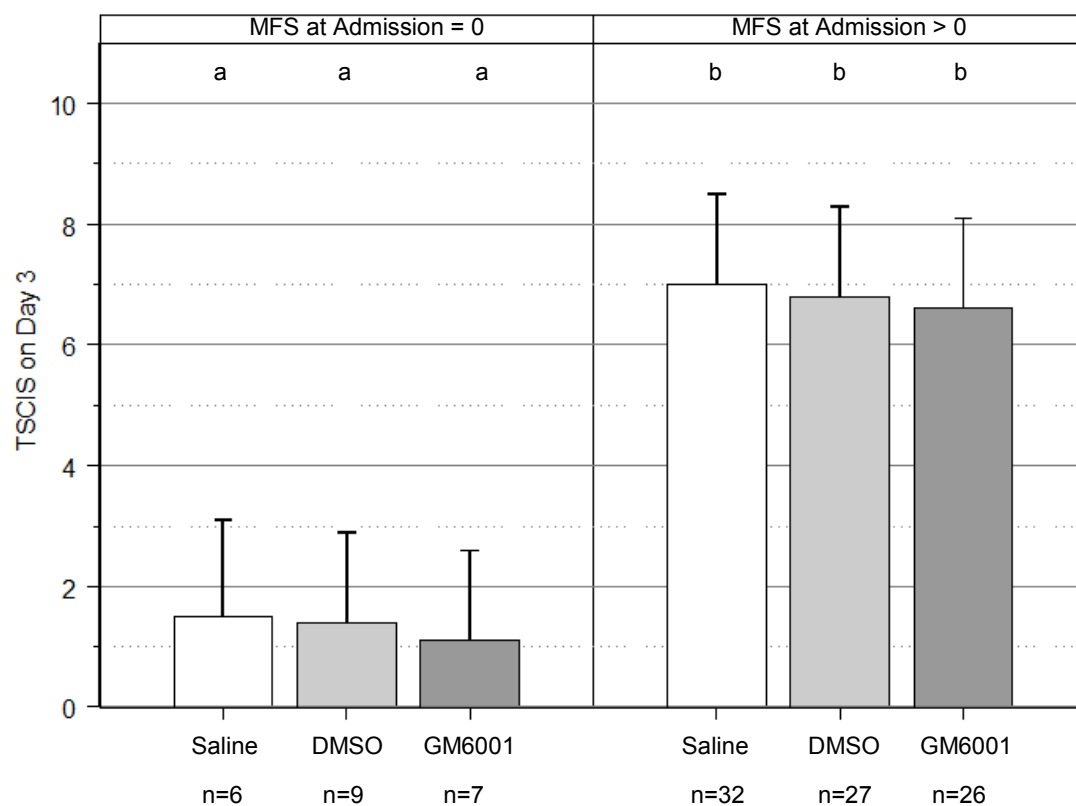


Supplemental Figure 2: The diameter of each of 6 drug delivery sites in dogs receiving 6-18 times the cumulative clinical trial dose of GM6001. Delivery site diameter appeared greatest 1 day after the administration of GM6001 (A) and qualitatively diminished by day 8 post-administration (B).

Supplemental Figure 3: Although values for MMP 2/9 activity in CSF tended to be higher for the dogs with spinal cord injuries (SCI; N = 40) than for healthy controls (N = 5), this difference was not significant ( $P = 0.3841$ ; t-test). Bars represent mean values; error bars represent the standard deviation.



Supplemental Figure 4: Bar graph of mean TSCIS scores on day 3 by treatment group, stratified by MFS score at admission (MFS = 0, left panel; or MFS > 0, right panel). There were no significant differences in TSCIS based on treatment group within each injury strata. See Figure 1 for description of boxes and whiskers. Bars with different letters differ significantly ( $P < 0.05$ ).



Supplemental Table 1: Frequency of adverse events and survival (died or euthanized) by treatment group. Injection site reactions were significantly associated with the study treatment, but no other adverse events were significantly associated with treatment. ND= not determined.

<b>Adverse Event</b>	<b>Saline Control</b>	<b>DMSO</b>	<b>Drug</b>	<b>P</b>
<b>Any</b>	29% (11/38)	28% (10/36)	55% (18/33)	0.0682
<b><i>Injection site reaction</i></b>	5% (2/38)	0% (0/36)	45% (15/33)	< 0.0001
<b><i>Gastrointestinal problem</i></b>	16% (6/38)	25% (9/36)	24% (8/33)	0.6097
<i>Anorexia</i>	0% (0/38)	3% (1/36)	0% (0/33)	ND
<i>Diarrhea</i>	10% (4/38)	8% (3/36)	15% (5/33)	ND
<i>Regurgitation</i>	0% (0/38)	3% (1/36)	0% (0/33)	ND
<i>Vomiting</i>	3% (1/38)	11% (4/36)	3% (3/33)	ND
<i>Vomiting + diarrhea</i>	3% (1/38)	0% (0/36)	0% (0/33)	ND
<b><i>Urinary Tract Infection</i></b>	5% (2/38)	3% (1/36)	3% (1/33)	1.0000
<b><i>Fever</i></b>	3% (1/38)	0% (0/36)	3% (1/33)	0.7588
<b><i>Other adverse events</i></b>	3% (1/38)	6% (2/36)	0% (0/33)	0.6451
<i>Seizures</i>	3% (1/38)	0% (0/36)	0% (0/33)	ND
<i>Pneumonia</i>	0% (0/38)	3% (1/36)	0% (0/33)	ND
<i>Cardiovascular</i>	0% (0/38)	3% (1/36)	0% (0/33)	ND
<b><i>Died or euthanized</i></b>	3% (1/38)	6% (2/36)	0% (0/33)	0.6451

Supplemental Table 2: Values of CSF MMP 2/9 activity in dogs with spinal cord injury were not significantly associated with signalment, duration of clinical signs, or MFS at the time of admission. Medians (and ranges) and P values derived from Wilcoxon-rank sum tests were reported for the categorical variables listed below.

<b>Variable</b>	<b>Median (Range) of MMP 2/9 Activity in CSF</b>		<b>P value</b>
<b>Age</b>	<b><math>\leq 5</math> Years (N = 29)</b> 528,300 (90,722 - 2,465,990)	<b><math>&gt; 5</math> Years (N = 13)</b> 623,624 (90,576 - 1,192,415)	0.8596
<b>Sex</b>	<b>Male (N = 22)</b> 562,403 (90,576 - 2,465,990)	<b>Female (N = 20)</b> 513,502 (90,722 - 1,192,415)	0.3449
<b>Neutered</b>	<b>Not neutered (N = 9)</b> 494,948 (94,915 - 1,455,369)	<b>Neutered (N = 33)</b> 555,397 (90,576 - 2,465,990)	0.7130
<b>Breed</b>	<b>Dachshund (N = 28)</b> 526,151 (90,576 - 1,455,369)	<b>Other (N = 14)</b> 637,540 (94,915 - 2,465,990)	0.3106
	<b>Chondrodysplastic (N = 39)</b> 528,300 (90,576 - 1,455,369)	<b>Other (N = 3)</b> 591,240 (503,002 - 2,465,990)	0.3051
<b>Duration of clinical signs prior to admission</b>			
	<b><math>\leq 12</math> hours (N = 12)</b> 524,524 (90,722 - 2,465,990)	<b><math>&gt; 12</math> hours (N = 30)</b> 541,849 (90,576 - 1,275,724)	0.8129
	<b><math>\leq 24</math> hours (N = 37)</b> 528,300 (90,576 - 2,465,990)	<b><math>&gt; 24</math> hours (N = 5)</b> 751,791 (207,229 - 775,929)	0.6551
<b>T2-weighted hyperintensity</b>			
	<b>Absent (N = 21)</b> 555,397 (94,915 - 2,465,990)	<b>Present (N = 11)</b> 629,071 (90,722 - 1,455,369)	0.7504
<b>MFS score at admission</b>			
	<b><math>\leq 2</math> (N = 23)</b> 528,300 (90,576 - 2,465,990)	<b><math>&gt; 2</math> (N = 19)</b> 555,397 (94,915 - 1,290,030)	0.8795

Supplemental Table 3: Baseline characteristics did not differ significantly between treatment groups among dogs with signs for duration of 12 hours or less. Panel a. summarizes continuous variables using medians (ranges) by group, with P values from Kruskal-Wallis tests; panel b describes categorical variables using proportions by group with P values from chi-squared testing. \* MFS = Modified Frankel scale; # TSCIS = Texas Spinal Cord Injury Score

***Change a. Continuous variables: Medians (range); P value from Kruskal-Wallis testing***

<b>Variable</b>	<b>Saline Controls (N = 11)</b>	<b>DMSO (N = 12)</b>	<b>GM6001 (N = 17)</b>	<b>P</b>
<b>Age (years)</b>	7 (4 to 13)	5 (3 to 10)	5 (3 to 14)	0.2454
<b>Duration of signs prior to admission (hours)</b>	12 (1 to 12)	11 (4 to 12)	9 (2 to 12)	0.8161
<b>MFS*</b>	1 (0 to 3)	2 (0 to 3)	1 (0 to 3)	0.9701
<b>TSCIS#</b>	2 (0 to 8)	4 (0 to 8)	3 (0 to 6)	0.9142

***Change b. Categorical variables: P values from chi-squared testing***

<b>Variable</b>	<b>Saline Controls (N = 11)</b>	<b>DMSO (N = 12)</b>	<b>Drug+DMSO (N = 17)</b>	<b>P</b>
<b>Sex</b>				
<b>Female</b>	73% (8/11)	42% (5/12)	24% (4/17)	0.0902
<b>Male</b>	27% (3/11)	58% (7/12)	76% (13/17)	
<b>Neutered</b>				
<b>No</b>	0% (0/11)	42% (5/12)	24% (4/17)	0.1638
<b>Yes</b>	100% (11/11)	58% (7/12)	76% (13/17)	
<b>Breed</b>				
<b>Dachshund</b>	91% (10/11)	58% (7/12)	82% (14/17)	0.3535
<b>Other</b>	9% (1/11)	42% (5/12)	18% (3/17)	
<b>Chondrodys-trophoid</b>	91% (10/11)	83% (10/12)	88% (15/17)	0.9567
<b>Other</b>	9% (1/11)	17% (2/12)	12% (2/17)	
<b>Level of Spinal Cord Injury</b>				
<b>T12-T13</b>	18% (2/11)	42% (5/12)	35% (6/17)	0.7350
<b>T13-L1</b>	36% (4/11)	33% (4/12)	29% (5/17)	0.9689
<b>L1-L2, L2-L3, 45% (5/11) or L3-L4</b>		25% (3/12)	35% (6/17)	0.5896
<b>T2-Weighted Hyperintensity (only available for 24 dogs)</b>				
<b>Absent</b>	62% (5/8)	83% (5/6)	40% (4/10)	0.2554
<b>Present</b>	38% (3/8)	17% (1/6)	60% (6/10)	



Supplemental Table 4: Baseline characteristics did not differ significantly among treatment groups among dogs with signs for duration of > 12 hours. Panel a. summarizes continuous variables using medians (ranges) by group, with P values from Kruskal-Wallis tests; panel b describes categorical variables using proportions by group with P values from chi-squared testing. \* MFS = Modified Frankel scale; # TSCIS = Texas Spinal Cord Injury Score

**Change a. Continuous variables: Medians (range); P value from Kruskal-Wallis testing**

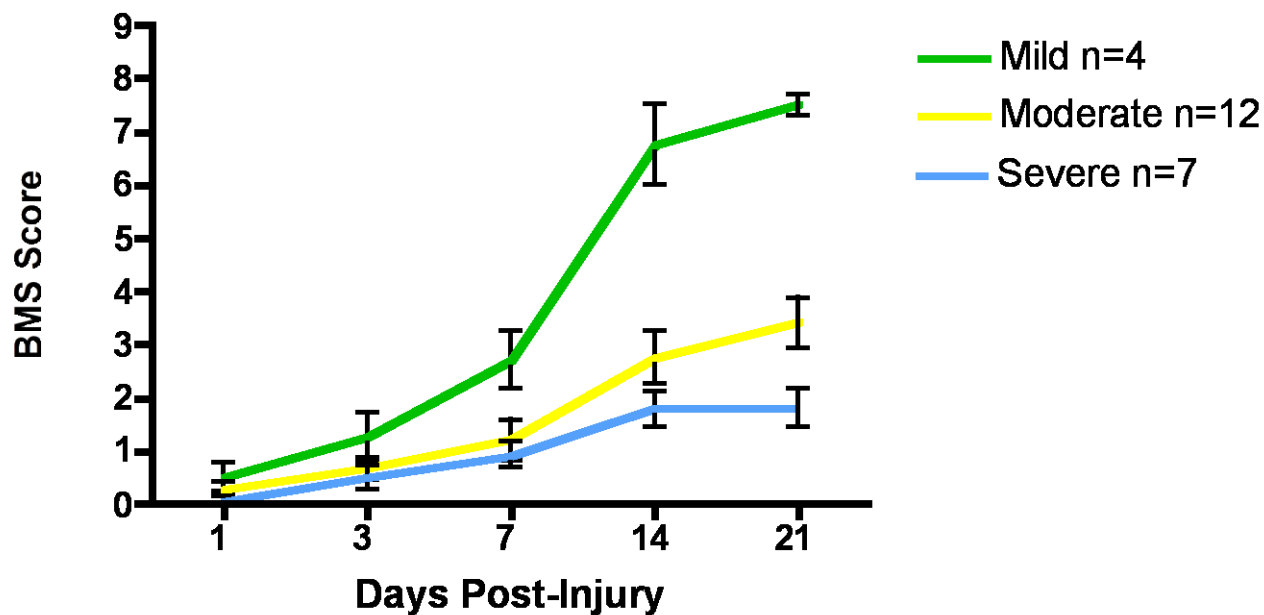
Variable	Saline Controls (N = 27)	DMSO (N = 24)	GM6001 (N = 16)	P
<b>Age (years)</b>	5 (2 to 10)	5 (3 to 13)	5 (2 to 11)	0.7593
<b>Duration of signs prior to admission (hours)</b>	24 (16 to 48)	24 (15 to 48)	24 (14 to 48)	0.4814
<b>MFS*</b>	2 (0 to 3)	2 (0 to 3)	2.5 (0 to 3)	0.6661
<b>TSCIS#</b>	4 (0 to 10)	4 (0 to 11)	4.5 (0 to 10)	0.5942

**Change b. Categorical variables: P values from chi-squared testing**

Variable	Saline Controls (N = 27)	DMSO (N = 24)	Drug+DMSO (N = 16)	P
<b>Sex</b>				
Female	56% (15/27)	58% (14/24)	56% (9/16)	0.9736
Male	44% (12/27)	42% (10/24)	44% (7/16)	
<b>Neutered</b>				
No	22% (6/27)	12% (3/24)	12% (2/16)	0.8296
Yes	78% (21/27)	88% (21/24)	88% (14/16)	
<b>Breed</b>				
Dachshund	63% (17/27)	62% (15/24)	88% (14/16)	0.3317
Other	37% (10/27)	38% (9/24)	12% (2/16)	
<b>Chondrodys-</b>				
trophoid	89% (24/27)	12% (3/24)	12% (2/16)	0.9139
Other	11% (3/27)	88% (21/24)	88% (14/16)	
<b>Level of Spinal Cord Injury</b>				
T12-T13	41% (11/27)	33% (8/24)	12% (2/16)	0.2792
T13-L1	26% (7/27)	21% (5/24)	38% (6/16)	0.9689
L1-L2, L2-L3, 33% (9/27) or L3-L4		46% (11/24)	50% (8/16)	0.4883
<b>T2-Weighted Hyperintensity (only available for 52 dogs)</b>				
Absent	62% (13/21)	74% (17/23)	62% (5/8)	0.8607
Present	38% (8/21)	26% (6/23)	38% (3/8)	

## SUPPORTING DATA UCSF

**Figure 1:** Production of a graded reproducible model of spinal cord injury in the mouse. Two-way repeated measures ANOVA showed significant interaction ( $p<0.0001$ ), significant effect of time ( $p<0.0001$ ) and significant effect of injury severity ( $p=0.0002$ ).

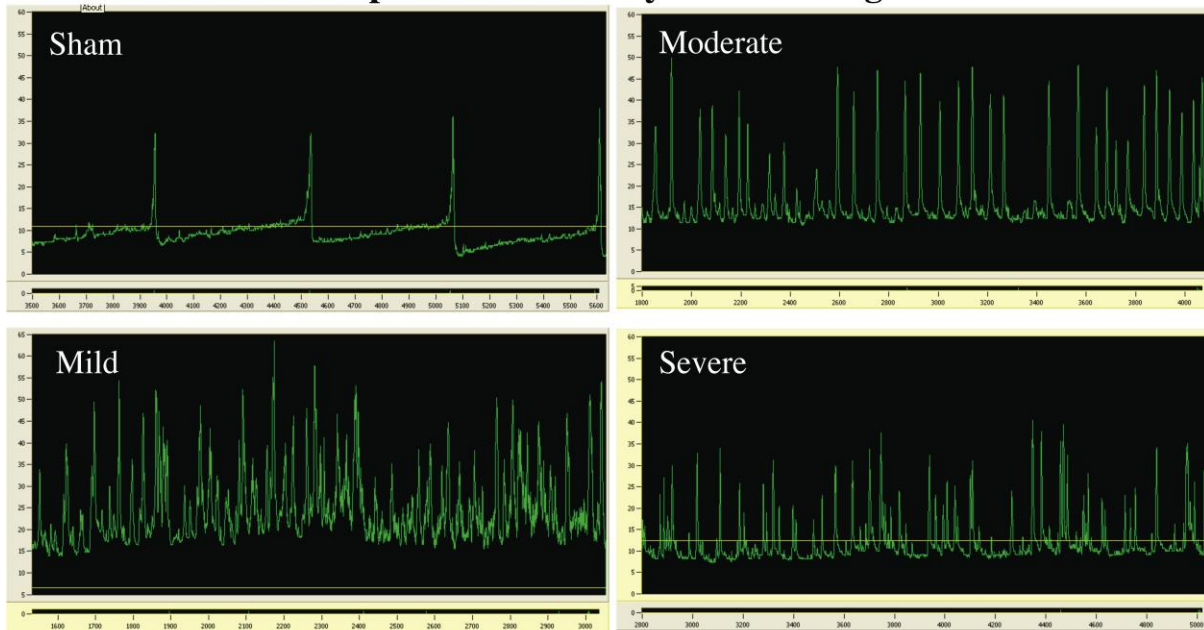


### Two way ANOVA

Interaction	$p<0.0001$
Time	$p<0.0001$
Injury Severity	$p=0.0002$

**Figure 2:** Uninhibited bladder contractions after mild, moderate, or severe spinal cord injury. Note the relative abundance of these contractions after mild injury relative to the more severely injured spinal cord.

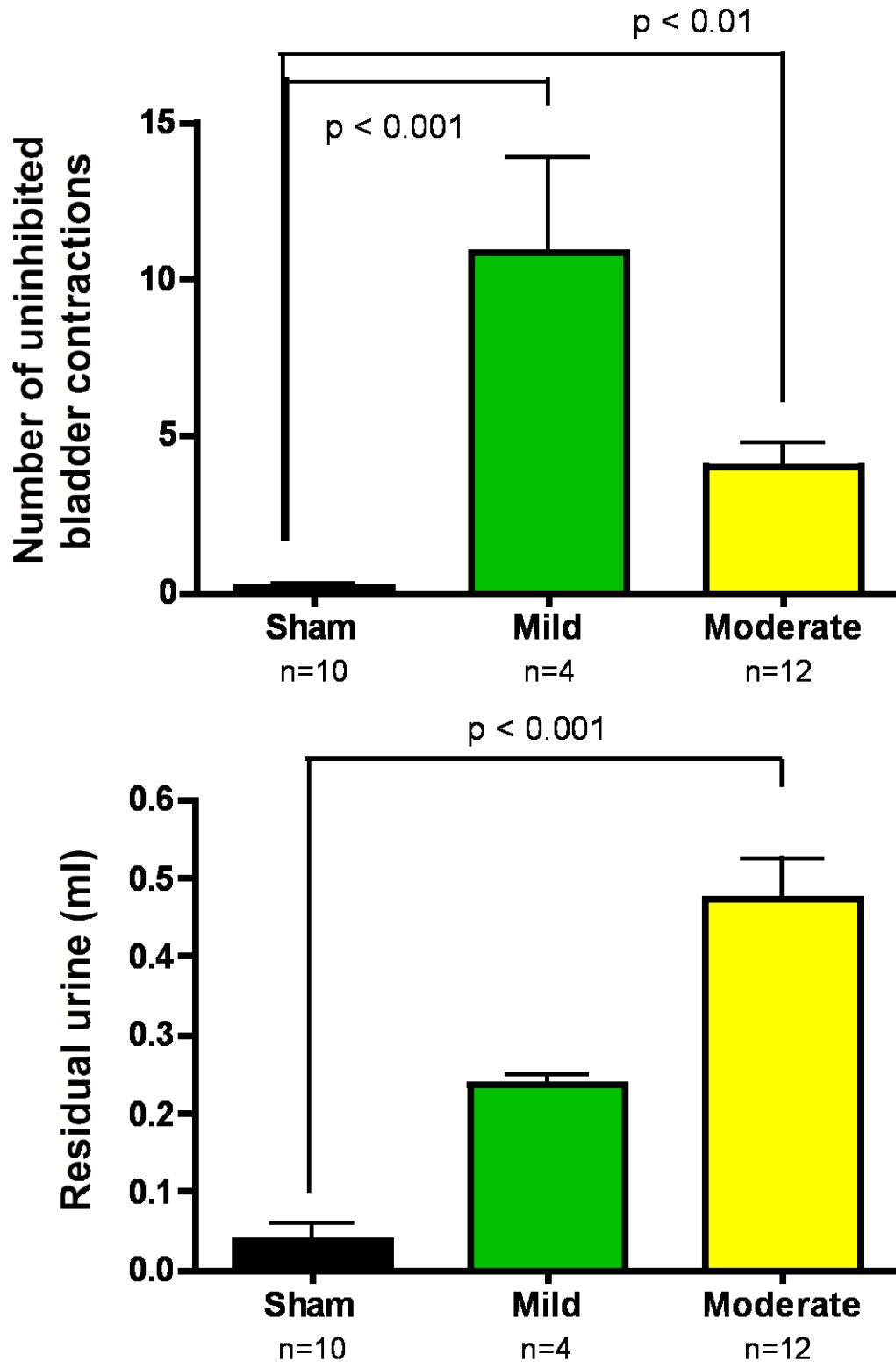
### Representative urodynamic tracings



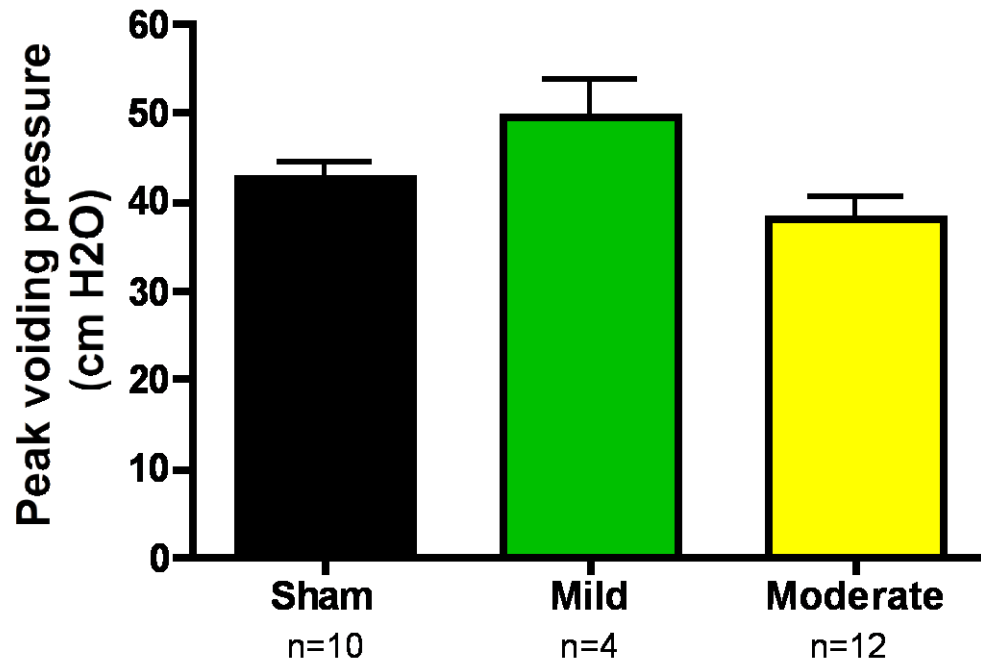
Cystometries were performed using restrained mice and a PE10 catheter with an infusion speed of 10 microliters/ minute.

**Figure 3:** Top Panel: Quantification of uninhibited bladder contractions after a mild and moderate spinal cord injury. Kruskal–Wallis test followed by Dunn’s Multiple Comparison Test, there were significantly increased numbers of bladder contractions after mild or moderate injury relative to the sham control group.

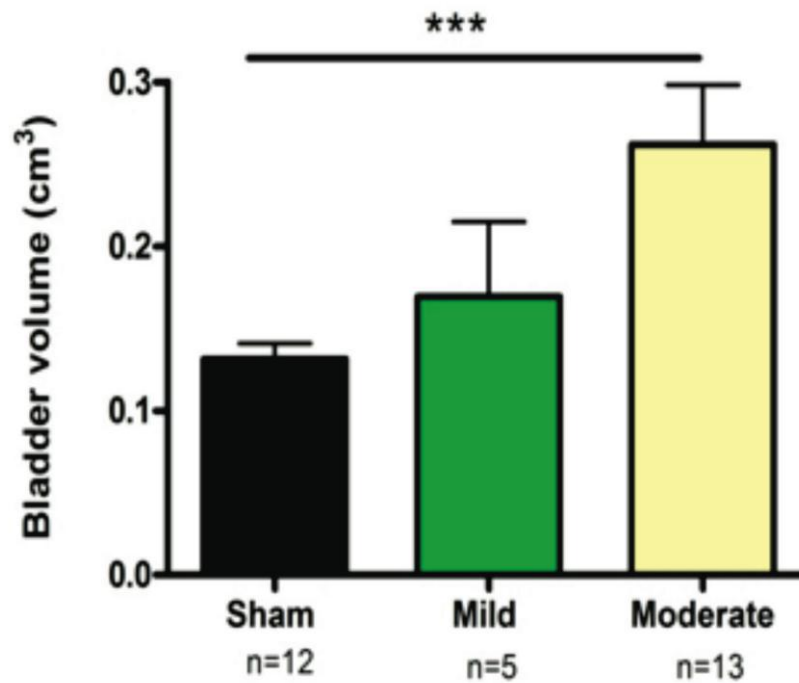
Bottom Panel: Quantification of residual urine. Kruskal–Wallis test followed by Dunn’s Multiple Comparison Test, moderate injury severity had more residual urine as compared to shams.



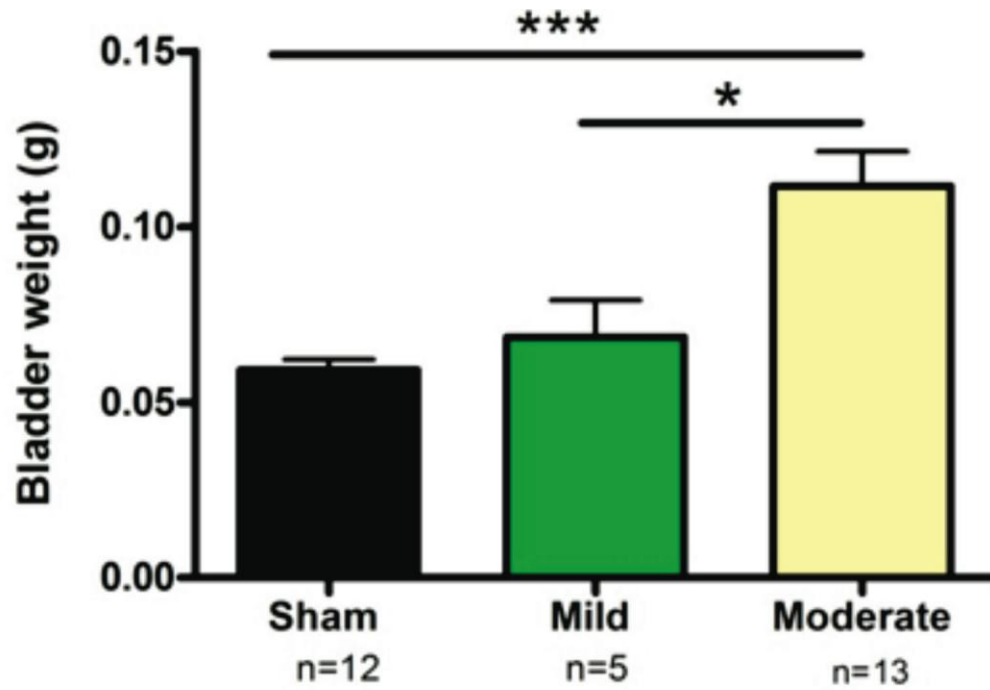
**Figure 4:** Peak pressure revealed no differences between groups (Kruskal–Wallis test,  $p>0.05$ ).



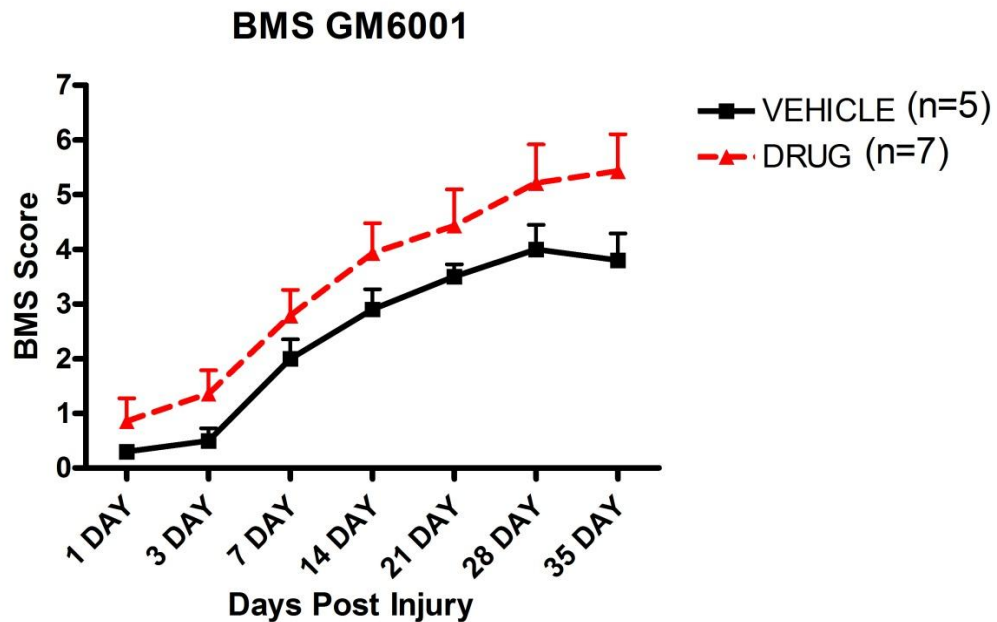
**Figure 5:** Bladder volume significantly increased after a moderate injury whereas there are no differences between mild and sham controls. Kruskal–Wallis test followed by Dunn’s Multiple Comparison Test, \*\*\*P<0.001



**Figure 6:** Bladder weight significantly increases after either mild or moderate injury compared to sham controls. Kruskal–Wallis test followed by Dunn’s Multiple Comparison Test, \*\*\* $P < 0.001$ , \* $P < 0.05$

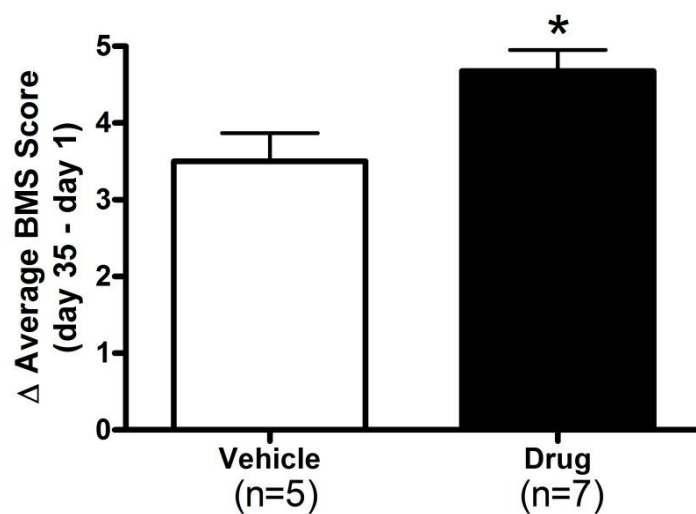


**Figure 7:** Male C57Bl/6 mice were subjected to a moderate spinal cord injury and treated with either GM6001 or 4% carboxy methyl cellulose beginning 8 hours post injury and every 12 hours thereafter for 3 days (route of delivery, intraperitoneal). While both groups improved locomotor function with time, there were no differences between drug and vehicle, based upon the BMS scale. Two-way repeated measures ANOVA of BMS score revealed the following:  $P = 0.58$  for interaction,  $P < 0.0001$  for time, and  $P = 0.16$  for treatment.

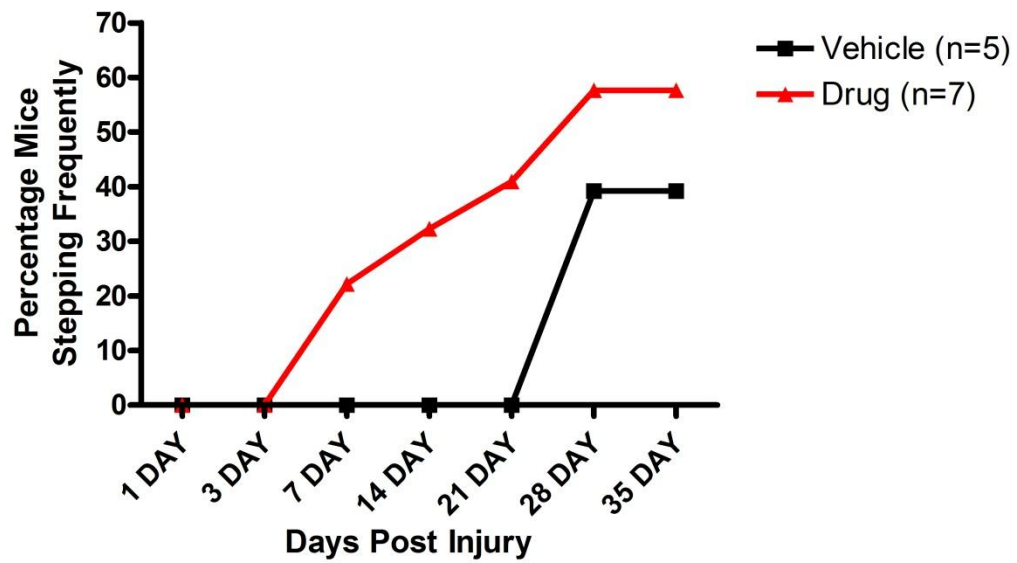




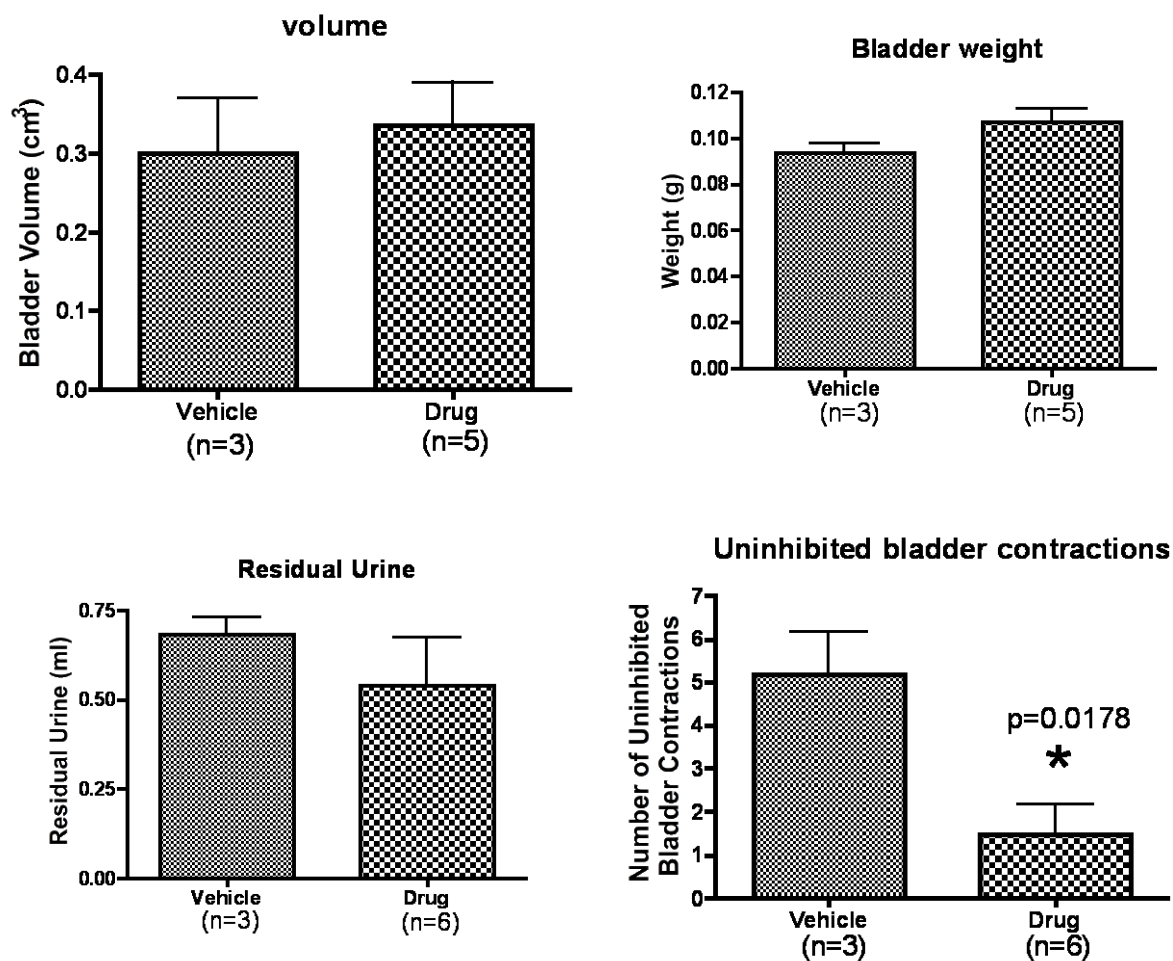
**Figure 8:** Comparison of initial BMS scores at 1 day versus 35 days, revealed a significant improvement in the GM6001-treated group. Student T-test, \* P=0.025



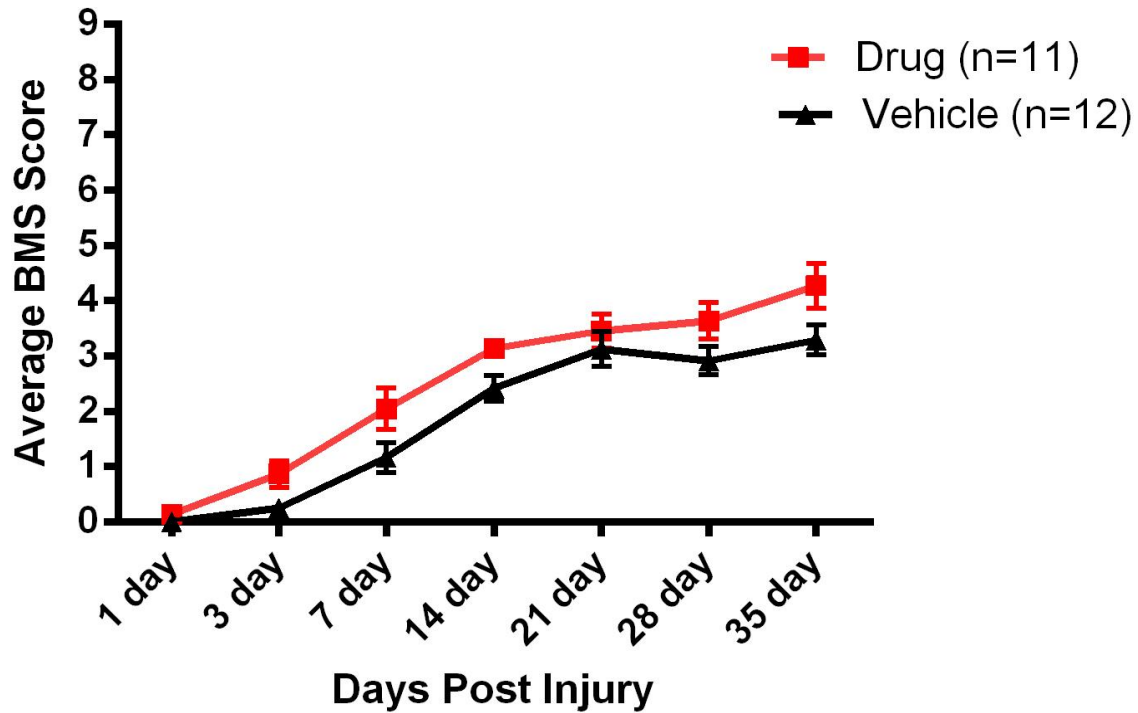
**Figure 9:** Evaluation of percentage of mice, subjected to a moderate spinal cord injury and GM6001 treatment show hindlimb stepping over time. Two way ANOVA, no significant interaction, significant effect of both treatment ( $P= 0.017$ ) and time ( $P= 0.015$ ).



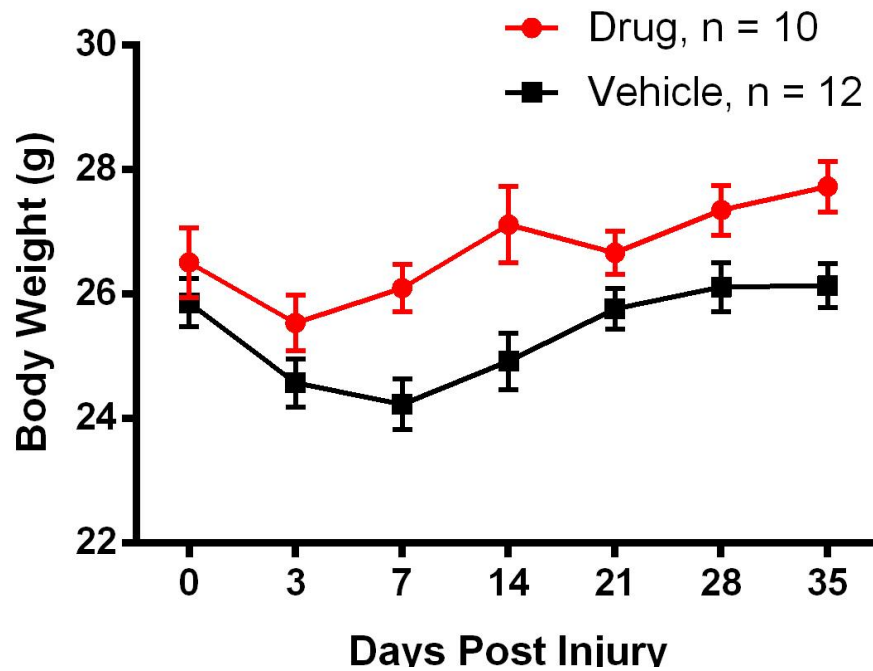
**Figure 10:** Analysis of bladder function after drug treatment in moderate spinal cord injury in mice. Cystometry was conducted in those animals that were also evaluated for neurologic function (Refer to Figure 7-9). There were signature reductions in number of uninhibited bladder contractions (Unpaired Students T-test).



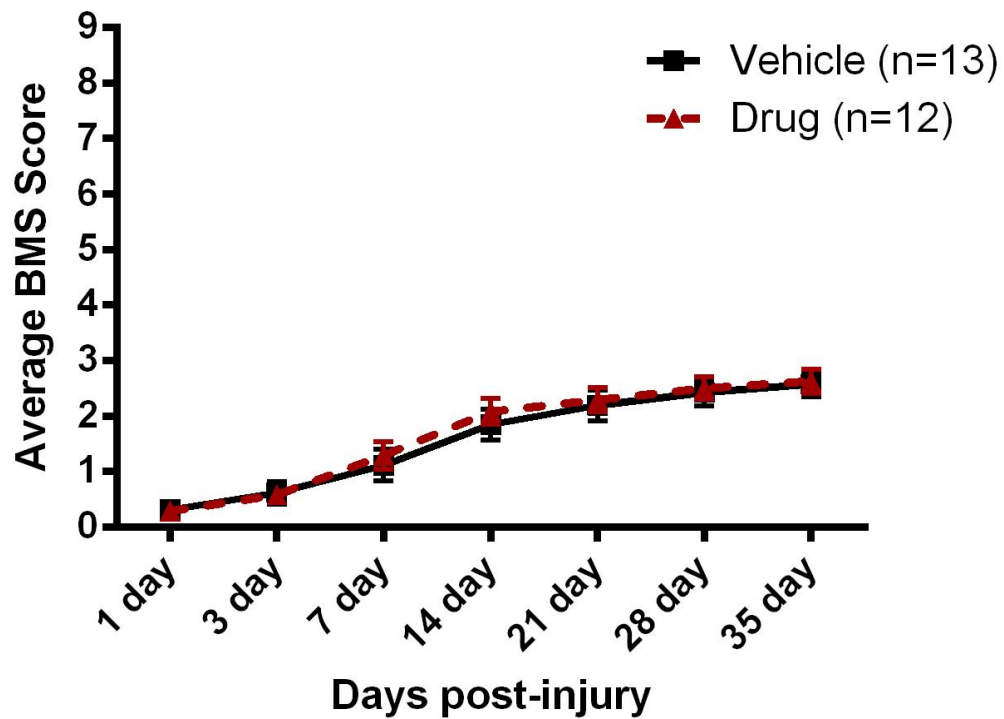
**Figure 11:** Male C57Bl/6 mice were subjected to a moderate spinal cord injury and treated with either GM6001 or DMSO (vehicle), injected subcutaneously, beginning 8 hours post injury and every 12 hours thereafter for 3 days. Not only did both groups improve locomotor function with time but GM6001 treated group performed better as compared to vehicle treated group, based upon BMS scale. Repeated measures Two-way ANOVA of BMS score revealed the following:  $P= 0.2453$  for interaction,  $P<0.0001$  for time, and  $P= 0.0397$  for treatment



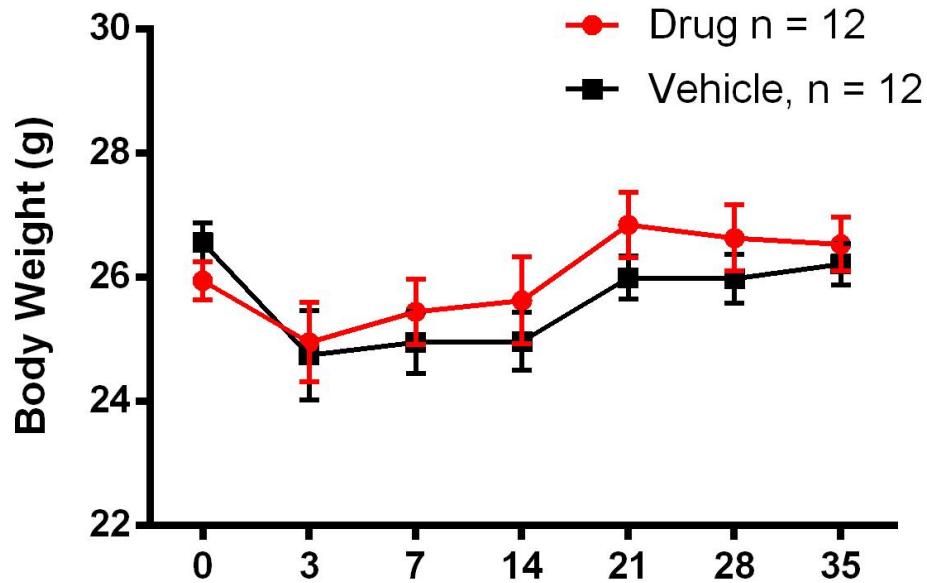
**Figure 12:** Male C57Bl/6 mice were subjected to a moderate spinal cord injury and treated with either GM6001 or DMSO (vehicle), injected subcutaneously, beginning 8 hours post injury and every 12 hours thereafter for 3 days. Not only did both gain weight with time but GM6001 treated group gained more weight as compared to vehicle treated group. Repeated measures Two-way ANOVA of weight revealed the following:  $P = 0.0676$  for interaction,  $P < 0.0001$  for time, and  $P = 0.0104$  for treatment.



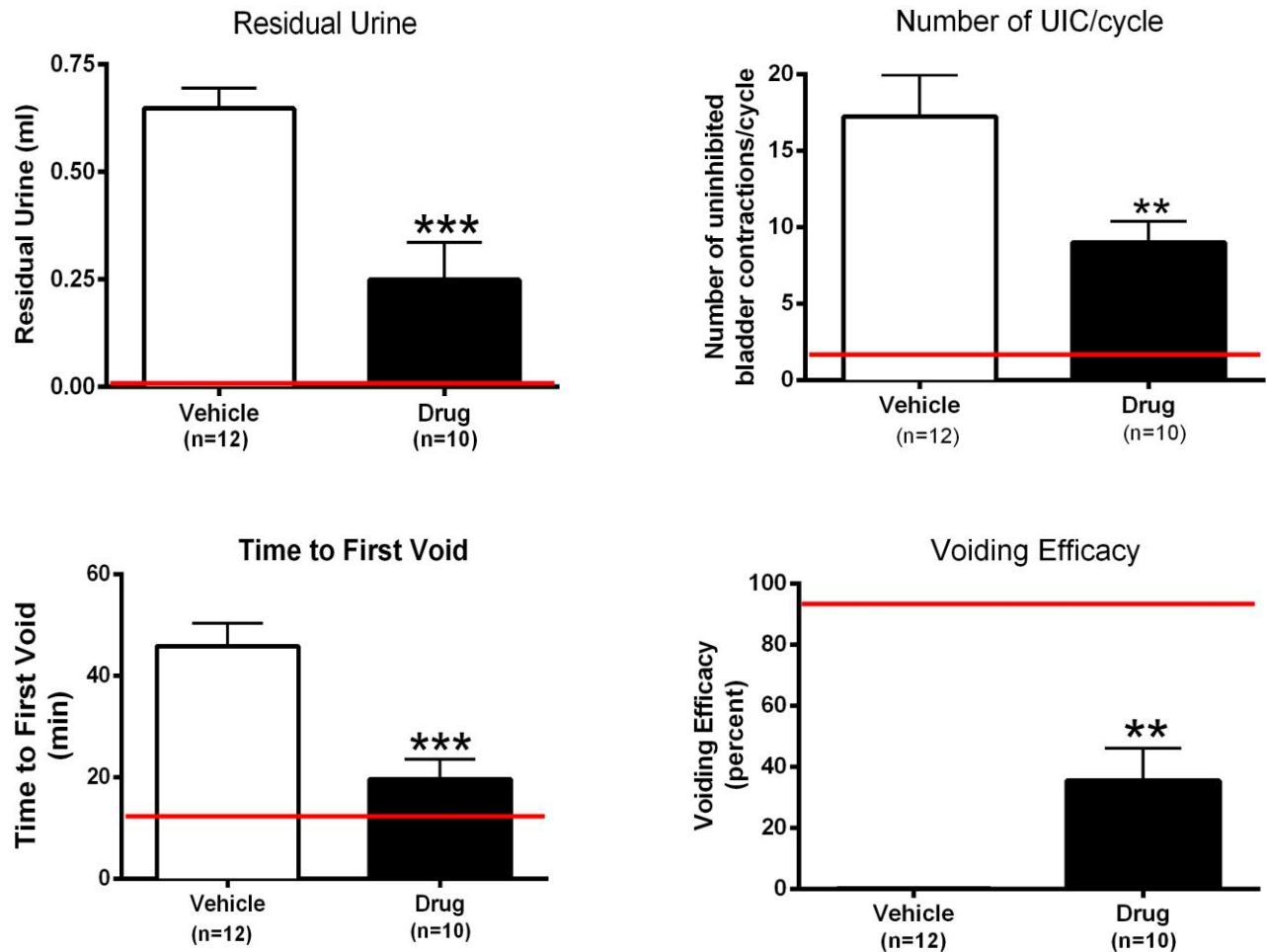
**Figure 13:** Male C57Bl/6 mice were subjected to a severe spinal cord injury and treated with either GM6001 or DMSO (vehicle) beginning 8 hours post injury and every 12 hours thereafter for 3 days. While both groups improved with time, there were no differences between drug and vehicle treated groups based upon BMS scale. Two-way repeated measures ANOVA of BMS score revealed the following:  $P = 0.9468$  for interaction,  $P < 0.0001$  for time, and  $P = 0.7530$  for treatment.



**Figure 14:** Male C57Bl/6 mice were subjected to a moderate spinal cord injury and treated with either GM6001 or DMSO (vehicle), injected subcutaneously, beginning 8 hours post injury and every 12 hours thereafter for 3 days. Not only did both gain weight with time but GM6001 treated group gained more weight as compared to vehicle treated group. Repeated measures Two-way ANOVA of weight revealed the following:  $P = 0.2662$  for interaction,  $P < 0.0001$  for time, and  $P = 0.5362$  for treatment.

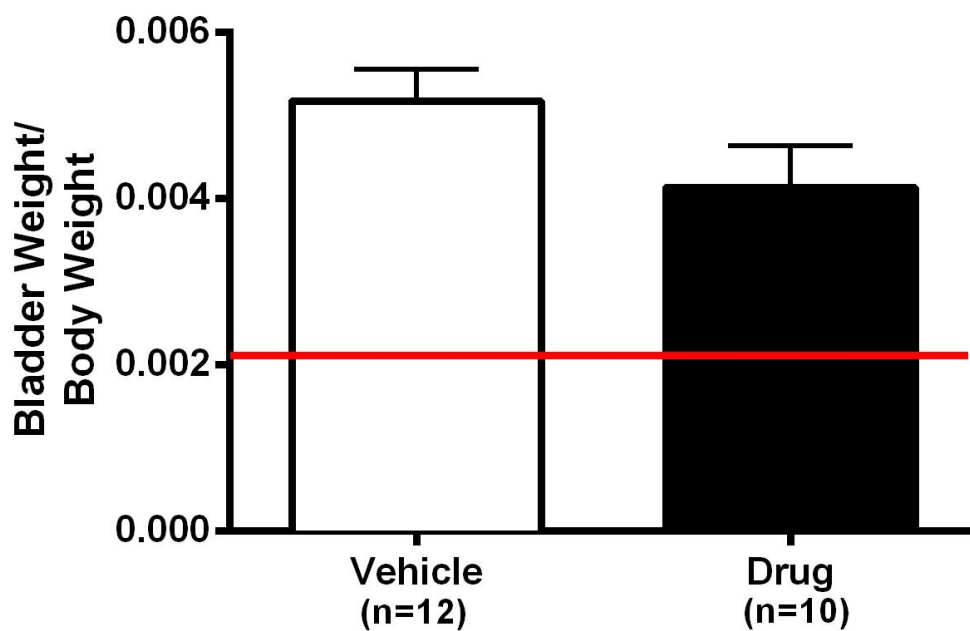


**Figure 15:** Urodynamic outcomes from awake cystometries of moderately injured mice treated with GM6001 or vehicle. Drug treated mice had better functional outcomes as compared to vehicle treated mice. They had less amount of urine retention (Unpaired two-tailed T- test,  $p=0.0004$ ), decreased number of uninhibited bladder contractions per cycle (Mann Whitney test,  $p=0.0067$ ), bladder voiding was observed at an earlier time point (Unpaired two-tailed T- test,  $p=0.0004$ ), and had better voiding efficacy (Mann Whitney test,  $p=0.0075$ ). Red lines in each graph indicate baseline values of uninjured male C57Bl/6 mice. Bars represent mean $\pm$ SEM.

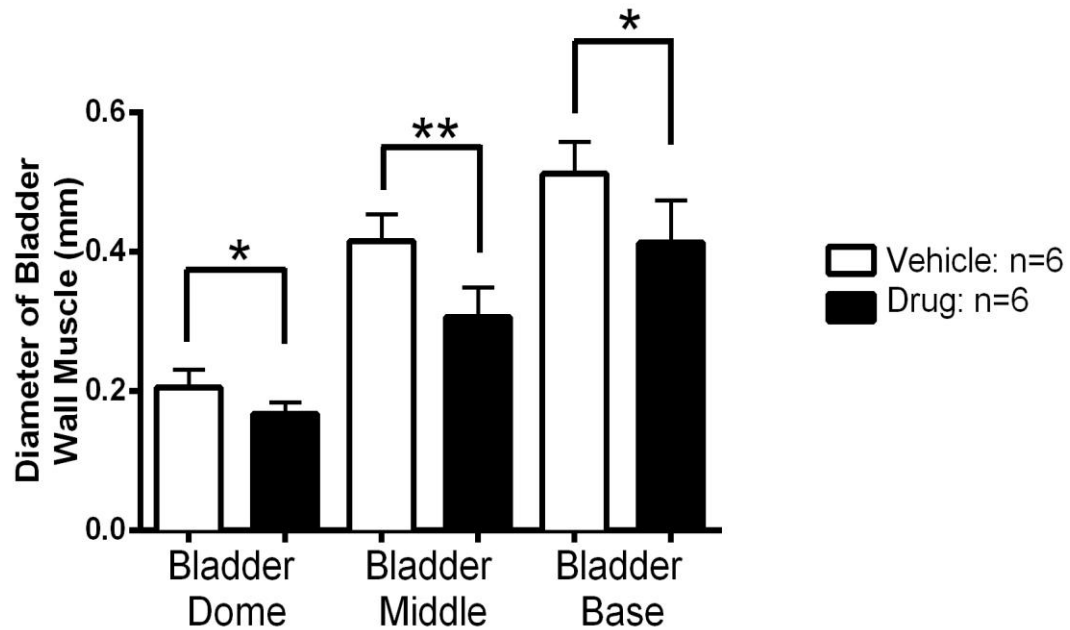




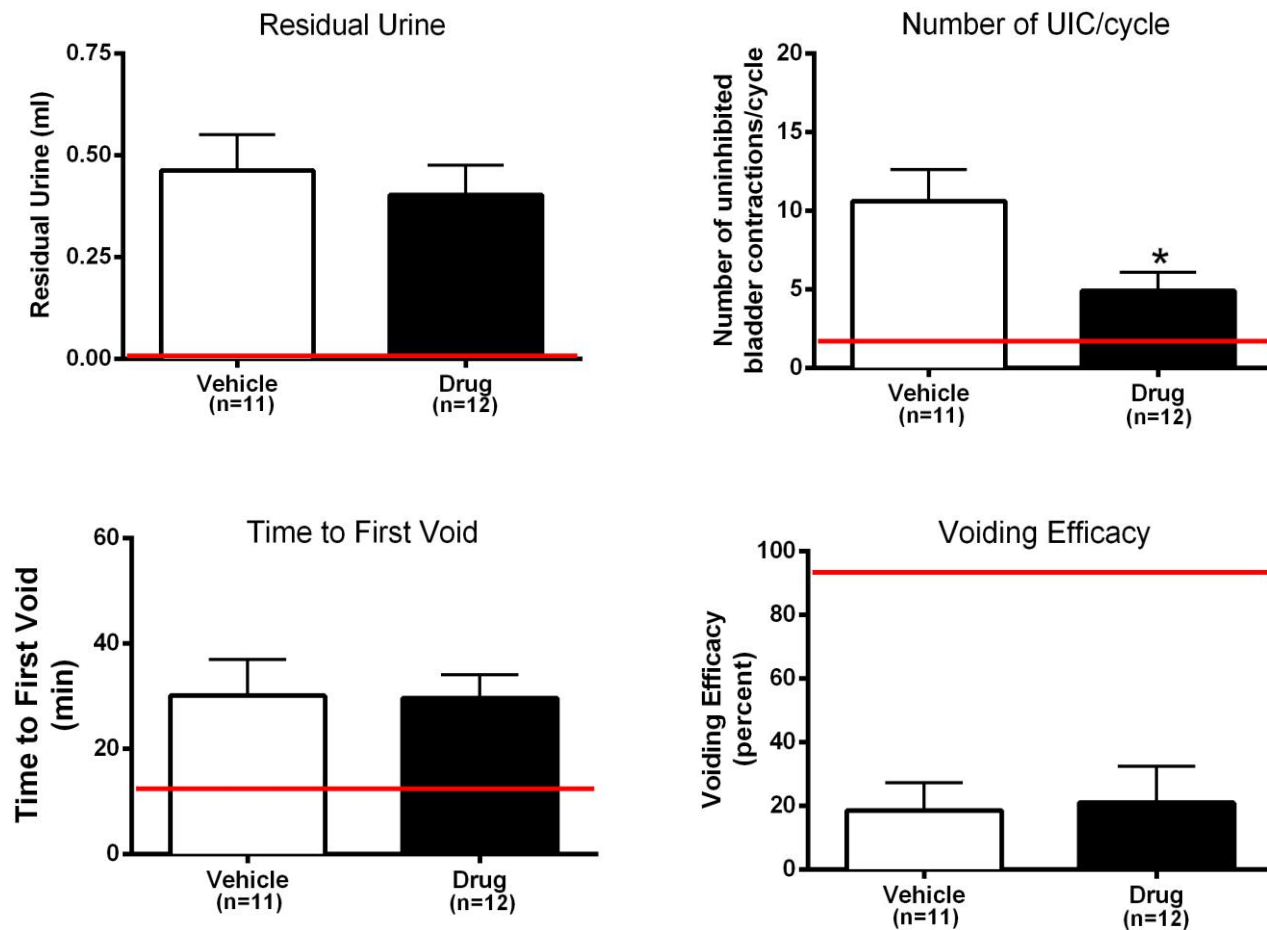
**Figure 16:** Even though drug treatment did not lead to significant decrease in bladder weight, there was a very strong trend (Mann Whitney test,  $p=0.0531$ ). Bars represent mean $\pm$ SEM.



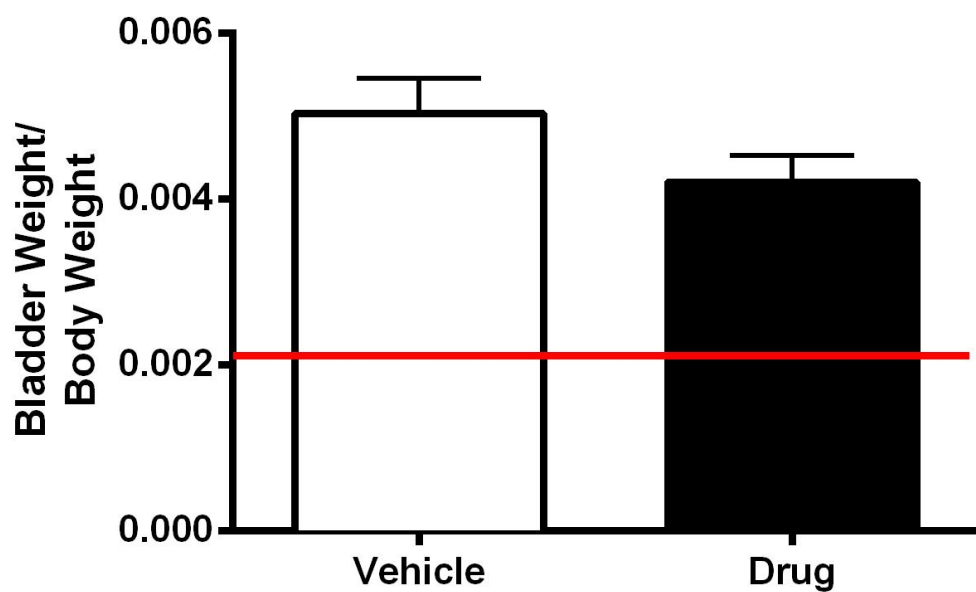
**Figure 17:** Bladder wall thickness was analyzed on sections stained with hematoxylin and eosin in three regions of the bladder (dome, middle and base). Drug treatment reduced wall thickness at each of these regions (Mann Whitney test,  $p=0.026$  for dome;  $p=0.0022$  for middle;  $p=0.0152$  for base). Bars represent mean  $\pm$  SEM.



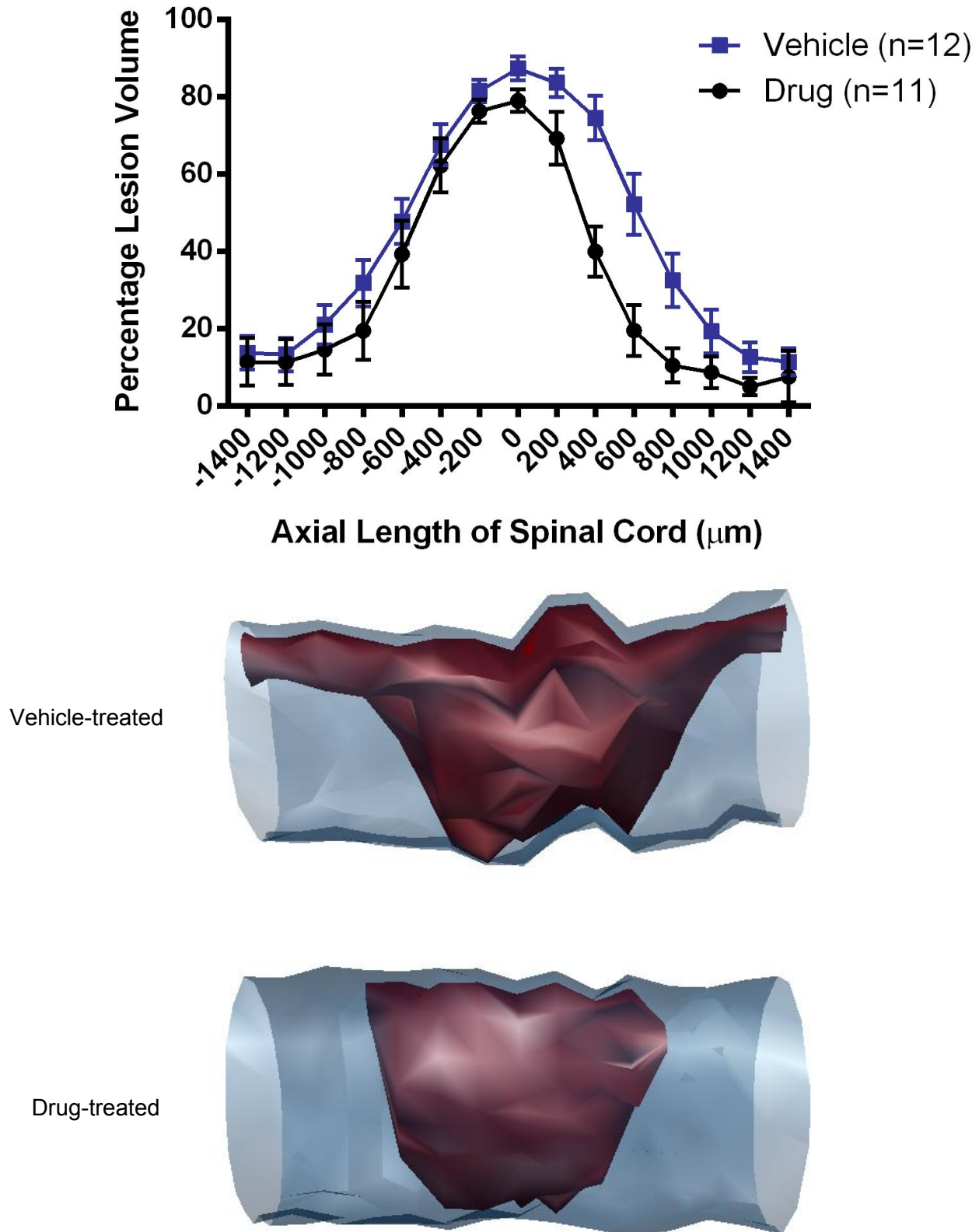
**Figure 18:** Urodynamic outcomes from awake cystometries of severely injured mice treated with GM6001 or vehicle. Drug treatment did not affect urine retention (Unpaired two-tailed T- test,  $p=0.6038$ ), decreased number of uninhibited bladder contractions per cycle (Unpaired two-tailed T- test,  $p=0.0210$ ), but had no effect on time to first void (Unpaired two-tailed T- test,  $p=0.9573$ ), or voiding efficacy (Mann Whitney test,  $p=0.4401$ ). Red lines in each graph indicate baseline values of uninjured male C57Bl/6 mice. Bars represent mean $\pm$ SEM.



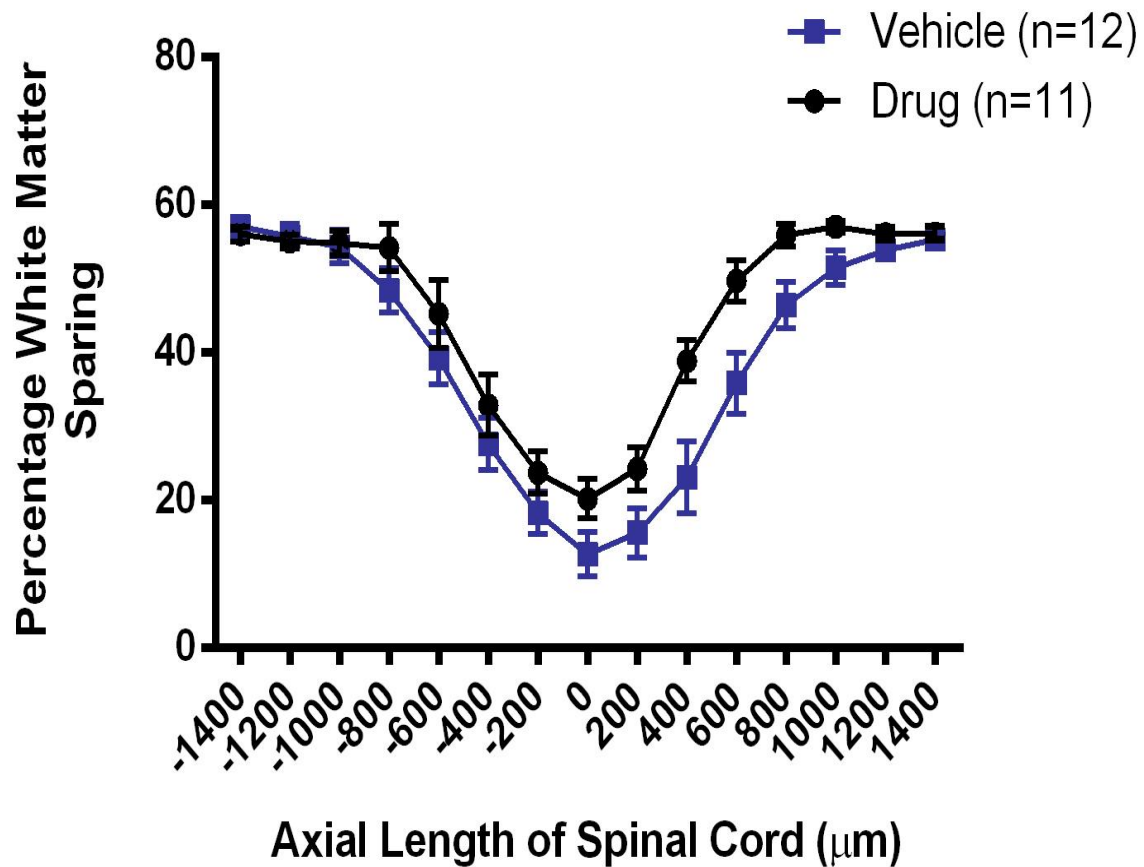
**Figure 19:** Bladder weights were analyzed from severely injured mice, treated with either vehicle or drug. Drug treatment did not lead to significant decrease in bladder weight (Unpaired two-tailed T test,  $p=0.1390$ ). Bars represent mean $\pm$ SEM.



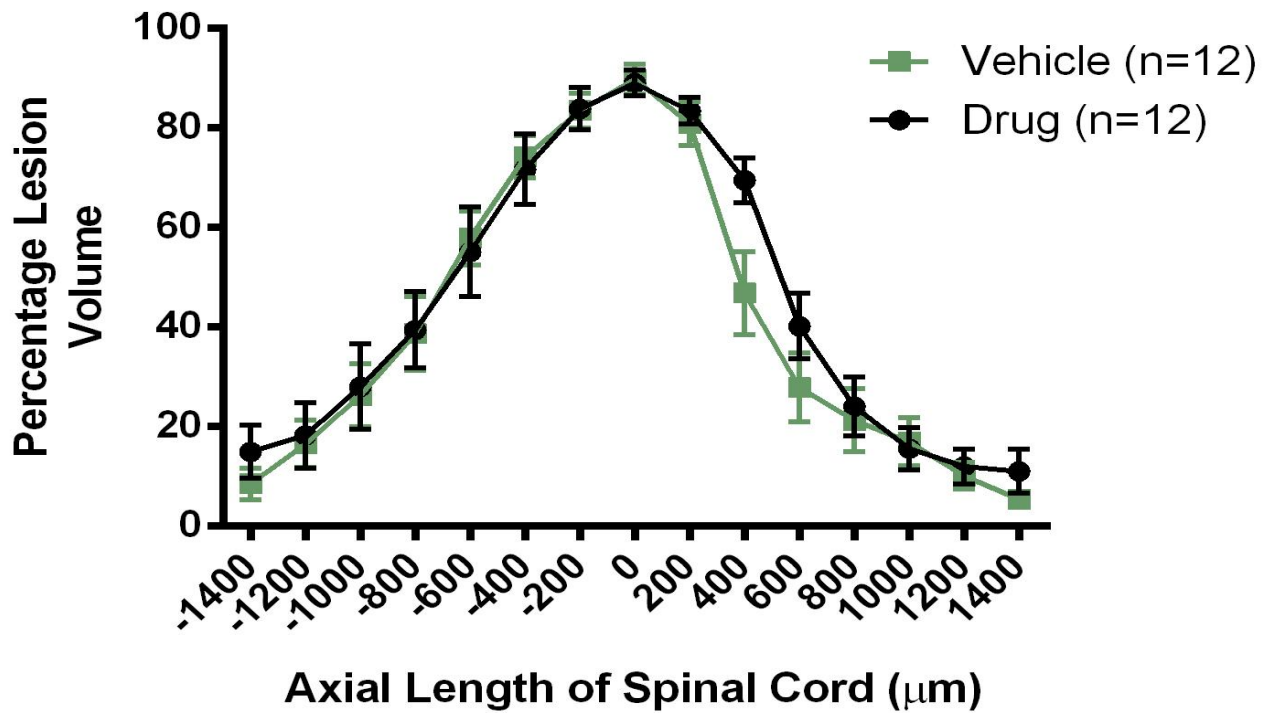
**Figure 20:** The rostral and caudal extent of lesion volume in moderately injured mice treated with GM6001 or vehicle was quantified at 200- $\mu$ m intervals spanning from 1400  $\mu$ m rostral to 1400  $\mu$ m caudal to the epicenter. The size of the lesion decreased at distances further removed from the epicenter and the drug treated group had smaller lesion as compared to the vehicle treated mice. Two-way repeated measures ANOVA of percentage lesion volume revealed the following:  $P=0.0714$  for interaction,  $P<0.0001$  for distance, and  $P=0.0001$  for treatment. 3D reconstruction images at the bottom show that the drug treated group has a smaller lesion as compared to vehicle treated group.



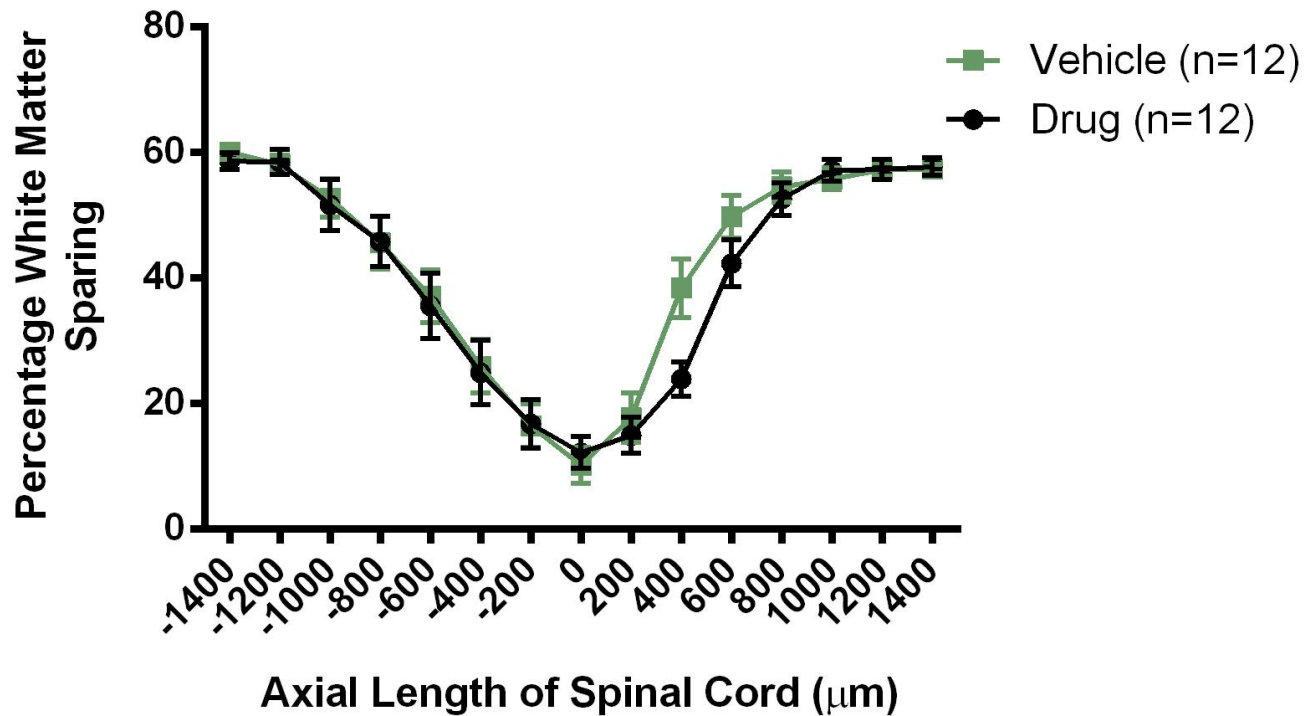
**Figure 21:** The rostral and caudal extent of spared white matter in moderately injured mice treated with GM6001 or vehicle was quantified at 200- $\mu$ m intervals spanning from 1400  $\mu$ m rostral to 1400  $\mu$ m caudal to the epicenter. Percentage spared white matter increased at distances further removed from the epicenter in both groups and the drug treated group had increased in spared white matter as compared to the vehicle treated mice. Two-way repeated measures ANOVA of percentage spared white matter revealed the following:  $P=0.1001$  for interaction,  $P<0.0001$  for distance, and  $P=0.0001$  for treatment.



**Figure 22:** The rostral and caudal extent of lesion volume in severely injured mice treated with GM6001 or vehicle was quantified at 200- $\mu$ m intervals spanning from 1400  $\mu$ m rostral to 1400  $\mu$ m caudal to the epicenter. Percentage lesion decreased at distances further removed from the epicenter in both groups but no differences were found between the groups in lesion volume. Two-way ANOVA, interaction,  $p=0.7712$ ; effect of distance,  $p<0.0001$ ; effect of treatment,  $p=0.1064$ .



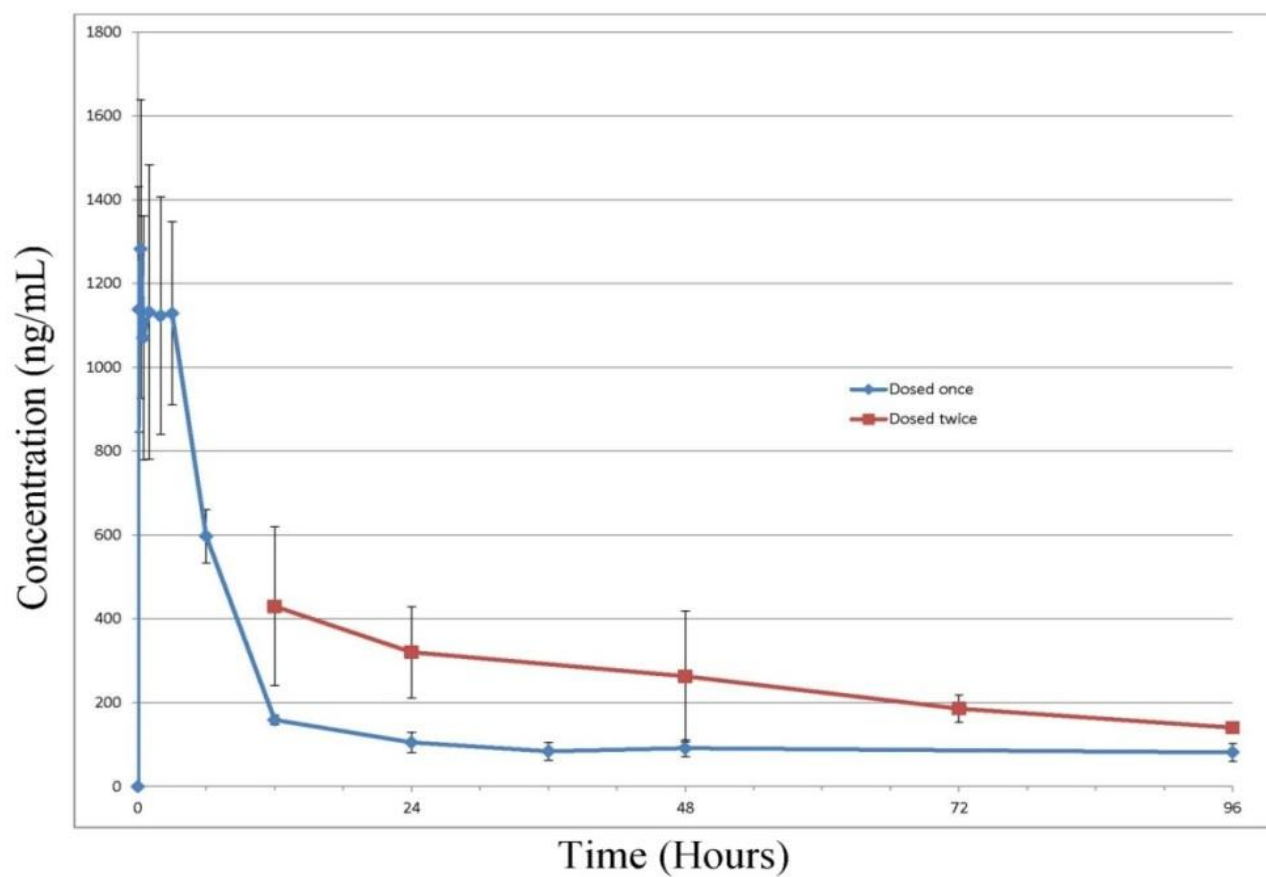
**Figure 23:** The rostral and caudal extent of spared white matter in severely injured mice treated with GM6001 or vehicle was quantified at 200- $\mu$ m intervals spanning from 1400  $\mu$ m rostral to 1400  $\mu$ m caudal to the epicenter. Percentage spared white matter increased at distances further removed from the epicenter in both groups but no differences were found between the groups in spared white matter. Two-way ANOVA, interaction,  $p=0.6069$ ; effect of distance,  $p<0.0001$ ; effect of treatment,  $p=0.1235$ .



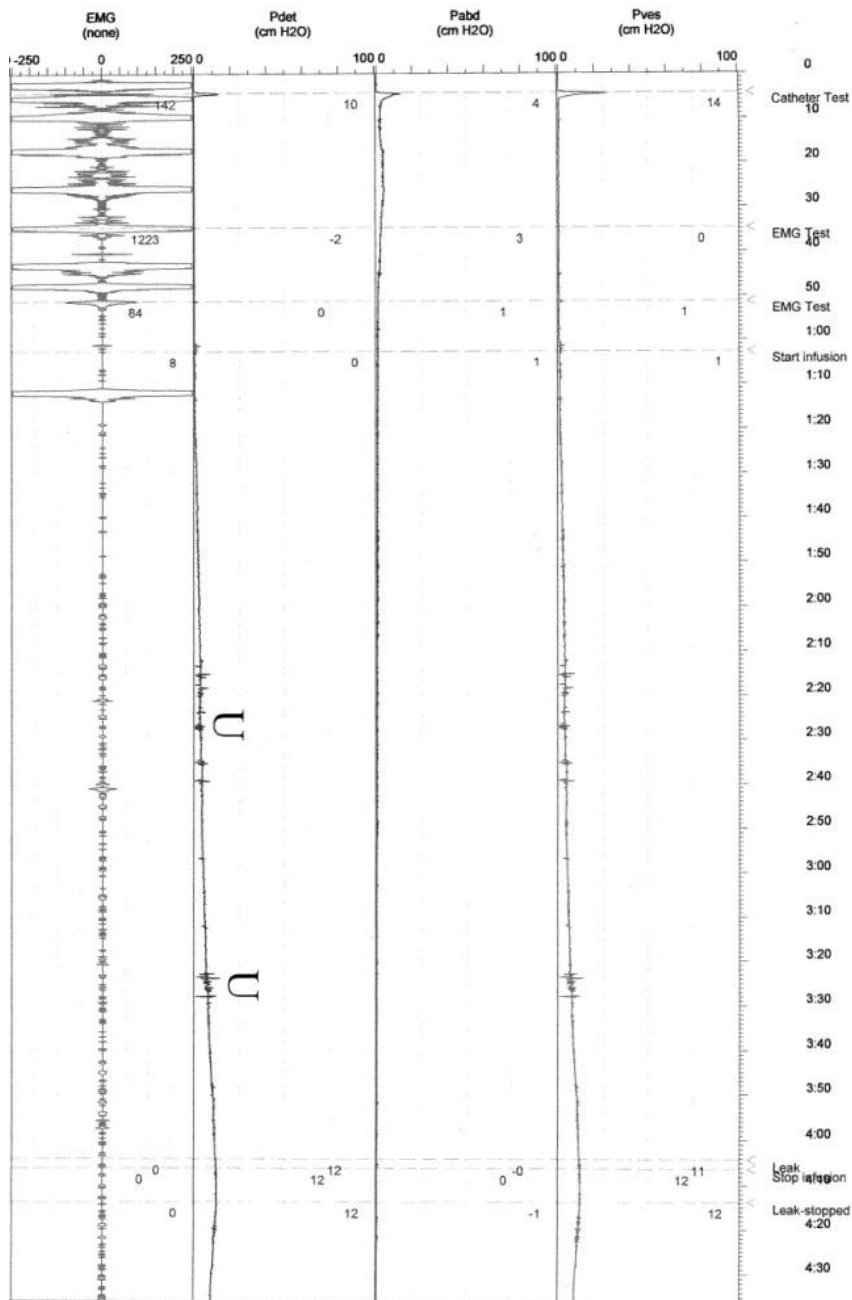


## SUPPORTING DATA- TAMU

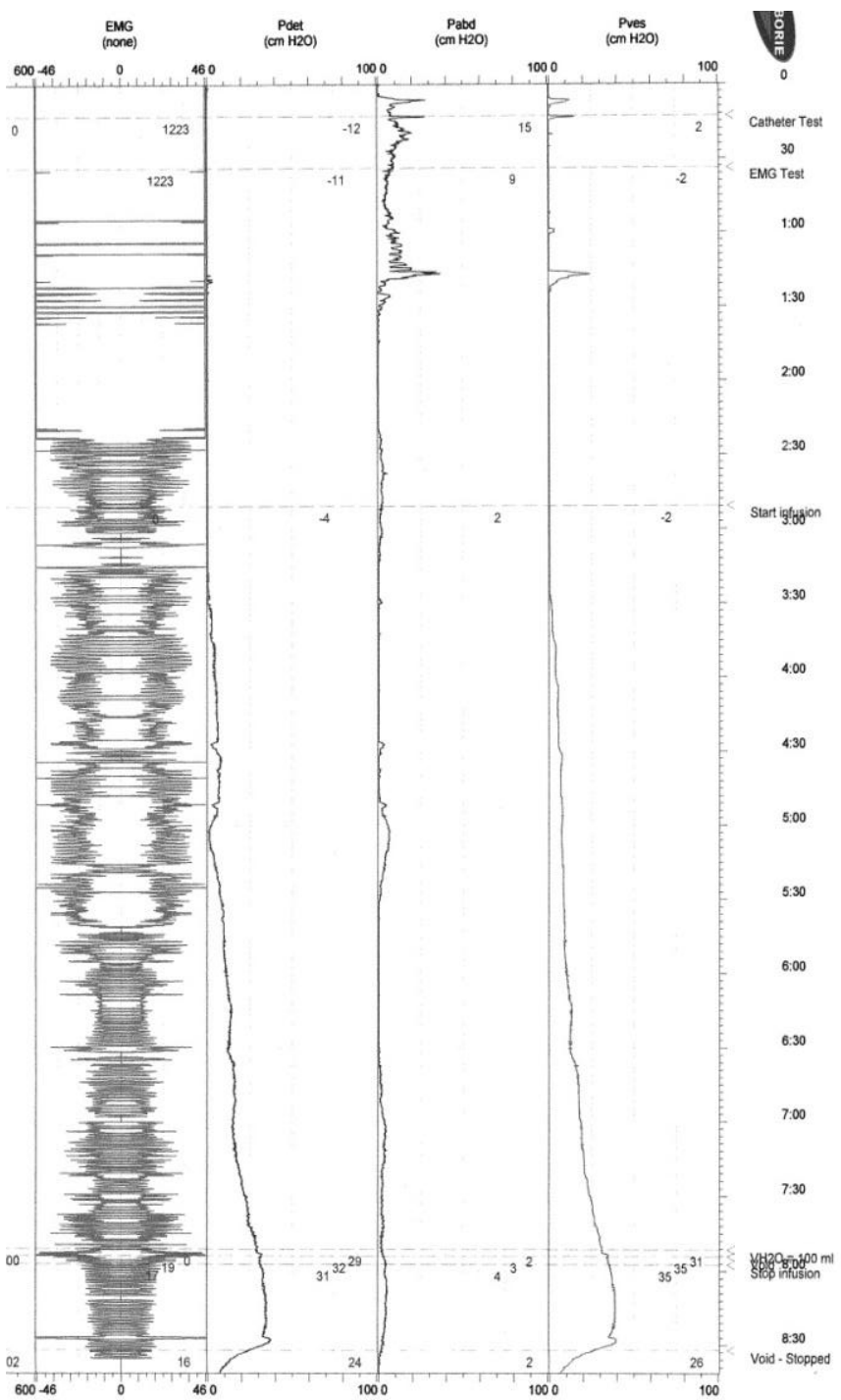
Figure 1: Plasma concentration of GM 6001 in 5 dogs dosed once at 100 mg/kg S.C. and 5 dogs dosed twice at a 12-hour interval. Data for the two-dose cohort was collected only at time points following the second dose.



**Figure 2. Cystometry 24 hours following spinal cord injury in a dog with intervertebral disk herniation. Note the absence of voiding, the presence of uninhibited bladder contractions (U), and the low leak point pressure (12 mm H<sub>2</sub>O)**



**Figure 3. Cystometry 3 days following Figure 2. Note the presence of a voiding reflex, the absence of un-inhibited bladder contractions, and the presence of anal EMG activity.**



**Figure 4: Quantification of MMP and ADAM activity in normal and SCI dogs. Dogs with SCI have significantly greater MMP-9 and ADAM-7 expression compared with healthy controls. No other MMPs or ADAMs were detected in the CSF of normal or SCI dogs. Dogs with severe SCI (MFS = 0) did not have significantly greater expression of MMP-9 or ADAM-7 than those with mild-to-moderate SCI (MFS = 3)**

